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# An Investigation of Motivational Dysfunctions in a Rat Model of Effort-Related Choice Behavior: Behavioral and Neurochemical Evidence for Possible Novel Pharmacological Treatments

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# An Investigation of Motivational Dysfunctions in a Rat Model of Effort-Related Choice Behavior: Behavioral and Neurochemical Evidence for Possible Novel Pharmacological Treatments

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Motivational symptoms such as fatigue, anergia, and amotivation are seen in depression, Parkinson's disease, schizophrenia, and other disorders. These symptoms are often left untreated by the most frequently prescribed antidepressants, which typically block serotonin transport. Considerable evidence implicates brain dopamine (DA) in the regulation of behavioral activation and effort-related aspects of motivation. Animal studies of effort-based choice are being used to provide formal models of motivational dysfunctions in humans. Drugs that block DA transport (DAT) are able to reverse the effort-related effects of the vesicular monoamine transport inhibitor tetrabenazine, a drug that blocks DA storage and depletes DA. Many of the existing DAT inhibitor drugs are either classic DA blockers such as cocaine, or drugs that also stimulate release of DA. These drugs can produce a number of undesirable side effects, including psychotic symptoms and abuse liability. Thus, there is a need to develop and characterize novel atypical DAT inhibitors that are relatively selective and have unique binding profiles. The completed studies discussed here focus on the behavioral and neurochemical characterization of several recently synthesized atypical DAT inhibitors, with the aim of identifying a novel family of drugs that may be useful for the treatment of motivational dysfunctions in humans, and the development of physiological markers of the selection of high-effort instrumental behavior. In recent years, human imaging and neurophysiological studies have had sought to identify the neural processes involved in motivation, psychomotor retardation, anergia, and lassitude in

depression and other disorders. Human studies have suggested that there is frontal electroencephalography (EEG) asymmetry in depressed patients, but physiological correlates of effort-related motivational dysfunction have not been identified in animals. To complement the investigation of effort-related impairments using pharmacological approaches, the final experiment involved the measurement of frontal cortex EEG activity of animals under pharmacological manipulation of DA transmission. Ultimately, the development of a physiological marker of effort-related dysfunction in a preclinical model may be critical for identifying the brain circuits involved in regulating these behaviors that can be readily translated to human studies. Together, results from these experiments may contribute to the identification of novel treatment options for motivational dysfunctions.

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## Chapter 1: Background

### *Role of Dopamine (DA) in Motivated Behaviors*

Motivation has been defined as the process that allows organisms to regulate their internal and external environment, and control the probability, proximity, and availability of stimuli (Salamone et al. 2017). On a day to day basis, organisms continually make effort-related decisions based upon cost/benefit analyses of reinforcement preference vs. response costs such as effort requirements. Central dopamine (DA) has been identified as a significant regulator of motivated behaviors in both animal and human studies. Behavioral paradigms such as the T-maze barrier and operant lever pressing tasks have been designed in order to measure effort-related decision making in rodents. These tasks are useful for assessing the activational aspects of motivation, described as the speed, vigor, and persistence of motivated behavior (Salamone and Correa 2002; Salamone et al. 2016a), and for characterizing how those functions can be manipulated under different experimental conditions, such as depletion or antagonism of DA. Behavioral activation in these tasks is regularly driven by incentives and cost/benefit strategies that assess the perceived benefits to be gained by exerting effort to obtain a highly valued stimulus. When DA transmission is reduced, the typical patterns of behavior are altered, and animals tend to select options with lower physical effort requirements. It has been suggested that these behavioral effects resemble symptoms associated with human depression such as anergia and fatigue (Salamone et al. 2016a).

Considerable evidence supports the role of DA in modulating the exertion of effort in rodents performing instrumental actions. For instance, interference with ventral striatal (i.e., accumbens) DA transmission preferentially affects high-effort instrumental activities elicited or supported by conditioned stimuli, whereas consummatory behaviors, low-effort instrumental

behaviors, and reinforcer preference remain intact (Aberman and Salamone 1999; Salamone and Correa, 2012). Drugs that can reliably reverse the low-effort bias induced by DA antagonists or depletions, such as DA transport (DAT) inhibitors, act to increase extracellular DA in the nucleus accumbens, which supports the notion that mesolimbic DA plays a direct role in activational or effort-related aspects of motivated behaviors.

*Motivational Aspects of Depression*

Major depressive disorder (MDD) is a psychiatric illness that affects over 300 million people worldwide (WHO, 2018). This number has increased steadily over the past several decades, and has been accompanied by a dramatic increase in the number of antidepressants that are being prescribed. The most widely recognized symptoms of major depression are emotional and cognitive in nature, but patients also exhibit lassitude, lack of self-reported energy, fatigue, psychomotor retardation, and motivational dysfunction (Stahl, 2006; Treadway and Zald, 2011; Fava et al. 2014; Salamone et al. 2016a), which are often the most difficult symptoms to treat. The most commonly prescribed medications for MDD are selective serotonin reuptake inhibitors (SSRIs), which tend to improve mood and reduce rumination and anxiety, but have little to no effect on symptoms of motivational dysfunction and fatigue (Papakostas et al., 2006; Pae et al., 2007; Cooper et al., 2014; Fava et al., 2014; Rothschild et al., 2014). Thus, these symptoms are often left untreated and can be severely debilitating.

Patients with depression, as well as Parkinson's disease and schizophrenia, show a low-effort bias when tested on effort-related choice procedures (Treadway et al. 2012; Yang et al. 2014; Chong et al. 2016; Barch et al. 2017), and it has been shown that the risk of depression in

individuals who report fatigue is two-fold greater compared to non-fatigued individuals (Corfield et al. 2016). These symptoms that involve amotivation and reductions in exertion of physical effort are commonly accompanied by elevated levels of pro-inflammatory cytokines such as IL-6, IL-1β, IFN-alpha, and TFN-α (Raison et al., 2006; Dantzer et al., 2008; Dowlati et al. 2010; Cattaneo et al. 2013), and several of these cytokines are upregulated as a result of exposure to chronic stress in animals (Miller et al. 2009; Lopresti et al. 2012). Rodent studies of effort-based choice have paralleled results from human research on motivational dysfunction, underlining their utility as animal models useful in determining possible treatment strategies for these symptoms (Dantzer et al. 2008; Nunes et al. 2014; Yohn et al. 2016b,d). These rodent studies focusing on modeling of effort-related motivation symptoms are consistent with the NIMH Research Domain Criteria (RDoC) approach, which emphasizes the importance of characterizing the neural processes that underlie the development of specific psychiatric symptoms.

*Pharmacological Models of Fatigue and Anergia in Rats*

Pharmacological agents that reduce DA transmission are used to treat symptoms of disorders such as schizophrenia and Huntington's disease, however, they have been shown to produce several undesirable side effects. For example, chronic treatment with DA antagonists such as haloperidol and other first-generation antipsychotics in schizophrenic patients is generally unsuccessful at treating negative symptoms such as lack of energy, fatigue, and amotivation (Harvey et al. 2016). In fact, drugs such as haloperidol can induce negative symptoms in healthy volunteers (Artaloytia et al. 2006). In addition, it has been reported that Huntington's disease patients treated with tetrabenazine (TBZ), a VMAT-2 inhibitor which depletes brain DA, tend to exhibit depressive symptoms including fatigue (Frank 2009, 2010;

Guay, 2010; Chen et al. 2012). When animals are administered TBZ in tasks measuring effort-related choice, high-effort baseline behavior is shifted towards a low-effort bias (Nunes et al. 2013; Randall et al. 2012, 2014; Yohn et al. 2015a,b). These effects are not dependent upon changes in food intake or preference, or discrimination of reinforcement magnitude, and do not resemble the effects of reinforcer devaluation or appetite suppressant drugs (Nunes et al. 2013; Yohn et al. 2015b; Randall et al. 2012, 2014). Rodent studies have also shown that a low-effort bias can be induced by inflammatory challenges (Nunes et al. 2014; Yohn et al. 2016d). The most commonly prescribed antidepressants, which inhibit the transport of serotonin (SSRIs or SERT inhibitors), do not reverse the effects of TBZ when co-administered, and actually tend to worsen them (Yohn et al. 2016a,b). However, administration of DAT inhibitors such as bupropion, GBR12909, lisdexamfetamine, and methylphenidate are able to reverse the effects of TBZ (Nunes et al. 2013, Yohn et al. 2016a,b; Salamone et al. 2016b) and of pro-inflammatory cytokines (Yohn et al. 2016c). These findings emphasize the need to identify and characterize a group of atypical DAT inhibitor compounds that are able to reverse the effects of DA antagonists/depletions or pro-inflammatory cytokines.

*Classical vs. Atypical Dopamine Transport Inhibitors*

Several drugs that inhibit DA uptake, such as bupropion, amphetamines, methylphenidate, and modafinil, have demonstrated the ability to partially reverse motivational dysfunctions in human studies of depression and other psychiatric disorders (Stotz et al. 1999; Hanna et al. 2006; Pae et al. 2007; Papakostas et al. 2006; Cooper et al., 2014). However, many of the existing drugs that are labeled as DAT inhibitors are either classical DA uptake blockers such as cocaine, or drugs that also stimulate release of DA, which can produce undesirable side

effects such as psychotic symptoms and abuse liability (Wilcox et al. 2002; Todtenkopf and Carlezon 2006; Ostlund et al. 2014; Dong et al. 2017). Psychomotor stimulants such as amphetamines and cocaine are categorized as "classical" DAT inhibitors due to their action at the DAT and their substantial effects on extracellular DA levels (Ferris et al. 2011; Schmitt et al. 2013; Tanda et al. 2013). When administered to rodents, both cocaine and amphetamine induce effects associated with recreational use and abuse liability, such as place conditioning (Tanda et al. 2013), conditioned DA release during self-administration (Phillips et al. 2003; Willuhn et al. 2010; Ostlund et al. 2014), and cue-induced craving (Dong et al. 2017). Taken together, this evidence suggests that while yielding positive effects in some studies focusing on motivational symptoms, there is probably a limited therapeutic utility in psychiatry for most classical DAT inhibitors. This highlights the importance of investigating the effort-related motivational effects of drugs that inhibit the uptake of DA, but may not be as likely to promote abuse and dependence.

A relatively new group of DA uptake inhibitors, known as atypical DAT inhibitors, has emerged over the last several years as a class of drugs that demonstrate distinct binding characteristics and different behavioral profiles from classical DAT inhibitors. These compounds are neither classified as cocaine-like DAT inhibitors nor amphetamine-like substrates and releasing agents due to their unique binding site and subsequent functional changes within the cell, resulting in a lack of cocaine-like subjective effects that result from binding (Schmitt et al. 2008; Schmitt et al. 2013; Kohut et al. 2014). The behavioral assessment of atypical DAT inhibitors such as benztropine, modafinil, and vanoxerine (GBR12909), has led to the discovery that not all inhibitors of DA uptake show signs of abuse liability to the same extent as the classical DAT inhibitors that are psychostimulants (Sogaard et al. 1990; Preti 2000; Woolverton

et al. 2001; Loland et al. 2008; Schmitt et al. 2008; Tanda et al. 2013). For example, while modafinil inhibits DAT (Schmitt and Reith 2011), and elevates extracellular DA as measured by microdialysis (Mereu et al. 2017) and human imaging studies (Volkow et al. 2009), this drug in humans does not induce a powerful euphoria or 'high' at doses that increase motivation and enhance task engagement (Muller et al. 2013). Some evidence indicates that modafinil binds to the DAT with different characteristics than cocaine (Schmitt and Reith 2011). However, the neurochemical bases for these different functional outcomes are still not completely understood.

Based upon all the studies reviewed above, it has become evident that there is a need to develop novel therapeutic strategies to treat motivational dysfunctions in humans. In order to be useful clinically, the behavioral and neurochemical profiles of novel drug targets need to be elucidated. The body of literature cited above outlines a class of drugs, known as atypical DAT inhibitors, which are currently being assessed for their ability to reverse motivational deficits induced by DA antagonists or depletions, and by pro-inflammatory cytokine challenges.

*Development of Physiological Measures of Effort-Related Aspects of Motivation*

Though the neural bases of the effort-related dysfunctions in psychopathology are still being characterized, considerable evidence indicates that the ability to exert effort and select high-effort options is dependent upon the functional integrity of forebrain circuits that involve mesolimbic DA inputs to nucleus accumbens, as well as the ventral pallidum, amygdala, and prefrontal cortex (Salamone et al. 1991, 1994; 2007, 2016a,b, 2018; Walton et al. 2003; Floresco and Ghods-Sharifi 2007; Mingote et al. 2008; Farrar et al. 2008, 2010; Winstanley and Floresco 2016). Systemic injections or local intra-accumbens infusions of drugs that block DA $D_1$ or $D_2$

family receptors can induce a low-effort bias in rats tested on effort-based choice procedures (Cousins et al. 1994; Nowend et al. 2001; Salamone et al. 2002; Sink et al. 2008; Warden et al. 2009; Nunes et al. 2010; Randall et al. 2014; Yohn et al. 2015b). While pharmacological manipulation of neural function remains an important tool in behavioral neuroscience, advances in molecular biology have led to the development of methods that allow for the silencing or activation of specific types of neurons, such as optogenetics and chemogenetics. Considerable evidence indicates that $D_1$ and $D_2$ receptors are predominantly localized on separate populations of neostriatal and accumbens medium spiny projection neurons, containing different peptide markers and distinct projection targets. Our laboratory has a long-range plan to investigate the role of accumbens neurons that express $D_1$ and $D_2$ receptors in effort-related function. Based upon circuitry models of basal ganglia, and the known signal transduction effects of stimulating $D_1$ and $D_2$ receptors (Mingote et al. 2008; Salamone et al. 2010; Farrar et al. 2010; Santerre et al. 2012; Nunes et al. 2013; Pardo-Garcia et al. 2019; Carvalho Poyraz et al. 2016), it is hypothesized that chemogenetic inactivation of accumbens neurons that express DA $D_1$ receptors will induce a low effort bias similar to that induced by TBZ.

As discussed above, pharmacological conditions that induce a low-effort bias in humans can alter effort-related choice in rats, and bias animals towards low-effort options (Salamone et al. 2016a,b,c; Yohn et al. 2016a,b,c), and these effects can be reversed by co-administration of several drugs that facilitate DA transmission. What is missing from this body of animal studies is the identification of a physiological marker that can be readily translatable to human clinical research. Recent studies have demonstrated that there are electroencephalographic (EEG) markers of frontal cortex activity that are characteristic of engagement in motivated behavior and anticipation of reinforcement, and that these markers are reduced in depressed people (Nelson et

al. 2018; Gheza et al. 2019). Moreover, these effects are associated with clinically rated lassitude in depressed individuals (Nelson et al. 2018). Generally speaking, these studies have implicated striatal and frontal cortical processes in approach motivation and effort-based decision making, which is consistent with animal studies in this area (Winstanley and Floresco 2016). Therefore, there is a need to employ EEG methods to aid in the development of physiological markers associated with altered DA transmission and effort-related dysfunction in preclinical animal models that can be readily translated to human studies.

*Present Work*

The following set of experiments was undertaken to investigate the behavioral and neurochemical evidence of motivational dysfunctions in rats, to uncover potential treatments for the effort-related motivational symptoms of depression and other disorders. The experiments discussed in **Chapter 2** aimed to evaluate the effects of the DA $D_2$ receptor antagonist haloperidol, chronically administered via subdermal minipump, in rats tested on effort-related procedures. Several experiments in this dissertation employed the vesicular monoamine transport (VMAT-2) inhibitor tetrabenazine to induce effort-related motivational impairments in rats (**Chapters 3-6**). Experiments described in **Chapters 3-5** aimed to characterize the effort-related and neurochemical effects of three recently synthesized atypical DA transport inhibitors. A critical goal of the final experiments in this dissertation (**Chapters 6-7**) was to employ a novel operant lever pressing task and EEG methods to aid in the development of physiological markers associated with altered DA transmission and effort-related dysfunction in preclinical animal models that can be readily translated to human studies. **Appendices 1-3** provide the results from studies involving compounds that do not act directly on DA transmission (i.e., serotonin and

kappa opioid receptors). Results from these studies will contribute to the development and discovery of novel treatments for treating anergia, fatigue, and motivational symptoms of depression, Parkinson's disease, and other disorders.

## Chapter 2: Evaluation of the effort-related effects of chronic administration of the DA $D_2$ receptor antagonist haloperidol via subcutaneous minipumps.

### 2.1 Introduction

Motivational/psychomotor symptoms such as response slowing, apathy, fatigue, and reduced exertion of effort are critical and debilitating features of major depressive disorder (Stahl 2002; Demyttenaere et al. 2005; Salamone et al. 2006; Treadway and Zald 2011; Fava et al. 2014). These symptoms, often classified as negative symptoms, are present in diverse psychiatric disorders in addition to depression, including schizophrenia, Parkinson's disease, and bipolar disorder (Caligiuri and Ellwanger 2000; Salamone et al. 2006, 2010; Friedman et al. 2007; Horan et al. 2015; Reddy et al. 2015; Fervaha et al. 2015; Treadway et al. 2015). Chronic treatment with DA antagonists such as haloperidol and other first-generation antipsychotics in schizophrenic patients is generally unsuccessful at treating negative symptoms such as lack of energy, fatigue, and amotivation (Kelley et al. 1999; Harvey et al. 2016). In fact, drugs such as haloperidol can induce negative symptoms in healthy volunteers (Artaloytia et al. 2006), and thus it has been hypothesized that a subset of the most common negative symptoms might be induced, at least partially, by chronic exposure to antipsychotic treatments.

In addition to physical symptoms of fatigue and amotivation, clinical reports indicate that chronic exposure to antipsychotics is often associated with adverse side effects such as dystonia, extrapyramidal symptoms, and sedation (Chouinard et al. 1993; Emsley et al. 2008; Essali et al. 2019). Drug tolerance or sensitivity have been commonly reported as effects of chronic antipsychotic use, yet these characteristics remain difficult to predict and to regulate due to myriad neurobiological factors (Li 2016). Treatment compliance issues are at the forefront of

schizophrenia and other psychiatric disorders due to undesirable side effects, impaired cognition, and/or a general lack of insight, which may ultimately lead to severe negative consequences (Xiao et al. 2015; Bitter et al. 2015; Phan 2016). For these reasons, it has become increasingly common for clinicians to prescribe long-acting injectable antipsychotics, including haloperidol decanoate, which is a depot formulation of the DA $D_2$ antagonist (Eklund and Forsman 1991; McEvoy et al. 2014; Brissos et al. 2015). Although long-acting depot formulations eliminate some of the medication adherence issues and decrease relapse rates (Brissos et al. 2015), the secondary negative effects of prolonged antipsychotic delivery can be debilitating and can severely impact daily functioning.

Fatigue and amotivation have been documented in rodents through the use of acute administration of DA $D_2$ antagonists in effort-related choice paradigms (Salamone et al. 1991; Salamone and Correa 2002, 2012a); however, the effects of chronic steady-state antipsychotic administration on effort-related processes are still unknown. Thus, assessing the behavioral changes that take place as a result of chronic DA $D_2$ antagonist administration in rats could provide insight regarding the development of secondary negative symptoms and the development of drug tolerance in schizophrenic patients (Gold et al. 2013; Fervaha et al. 2013; Barnes et al. 2013; Kring and Barch 2014; Barch et al. 2014; Reddy et al. 2015). The use of programmable infusion pumps has allowed researchers to implement continuous and controlled drug delivery which closely resembles long-acting and slow-release formulations of clinical drug therapies (Abe et al. 2010; Tan et al. 2011). The studies discussed below assessed the effects of chronic DA $D_2$ receptor blockade via subdermal haloperidol infusions on effort-related choice behavior, and the possible development of drug tolerance, or substantial carryover into the washout period.

## 2.2 Materials and Methods

*Animals*

Adult male Sprague Dawley rats (Envigo Sprague Dawley, Indianapolis, IN, USA; weights 275-299 g upon arrival) were pair-housed in a colony maintained at 23°C, with a 12-h light/dark cycle (lights on 07:00). Rats were food deprived to 85% of their free-feeding body weight for operant training and were fed supplemental chow as needed, allowing modest growth throughout the experiment. Water was available ad libitum in home cages. Animal protocols were approved by the University of Connecticut Institutional Animal Care and Use Committee, and the studies were conducted according to National Institutes of Health (NIH) guidelines.

*Behavioral Procedures*

Behavioral sessions were conducted in operant chambers (28 x 23 x 23 $cm^3$; Med Associates, Fairfax, VT) with 30 minute sessions 5 days/week. Rats were initially trained to lever press on a continuous reinforcement FR1 schedule (high-carbohydrate 45 mg pellets, Bio-Serv, Frenchtown, NJ) and then shifted to the FR5 schedule. Rats in **Exp 2.1** (n=15) remained on the FR5 schedule for four to five weeks, until a stable baseline was reached. Rats in **Exp 2.2** (n=17) underwent the same initial training, and after four weeks of FR5, were shifted to the concurrent FR5/chow feeding choice procedure for up to four additional weeks. Weighed amounts of laboratory chow (Laboratory Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO; typically 15-20 g) were concurrently available on the floor of the chamber during the FR5/chow feeding choice task sessions. At the end of each 30 minute session, rats were immediately removed from the chambers, number of lever presses was recorded, and the amount

of chow consumed was determined by weighing the remaining food (including spillage from a tray beneath the floor of the chamber). On baseline and drug treatment days, rats consumed all of the operant pellets that were delivered during each session. After a stable baseline was reached on the FR5 or FR5/chow procedure, rats in both experiments underwent a surgical procedure.

*Surgeries*

All rats underwent a surgical procedure for implantation of iPRECIO infusion pumps (iPRECIO SMP-200, Alzet). Pumps were stored at 45°C for 36 h prior to the surgery in a heating cabinet. On the day of surgery, the temperature was reduced to 37°C. Antiseptic technique was used and all instruments were cleaned and autoclaved prior to the procedure. Anesthesia was induced with 4-5% isoflurane in oxygen (and maintained in 2-3% isoflurane). The desired incision site was shaved, and betadine solution was used to disinfect the shaved area. Local analgesia (topical lidocaine) was applied at the incision site. Using a size 10 scalpel blade, a 4-cm midline incision was made approximately 8 cm from the top of the head. Blunt dissection was used, creating a pouch under the skin from the point of incision to the caudal area on the rat's right side, by separating the skin from the underlying tissue with hemostats. The pump was then removed from the heating cabinet, filled with sterile 0.9% saline, and the outlet tube was cut to 4 cm prior to being placed within the pocket. Non-absorbable nylon sutures (5-0) were used to attach the pump to the muscle layer. A blunt scissor was used to form a small tunnel for the outlet tube between the muscle and skin layers in the rostral direction. The tube was then sutured to the muscle ~1 cm from the tip. The skin incision was closed using 5-0 non-absorbable suture with 5-8 simple interrupted stitches or metal wound clips. After surgery, the rats were single-housed for a recovery period of at least seven days.

*Behavioral Pharmacology Experiments*

These experiments assessed the effects of continuous infusion of haloperidol vs. vehicle on performance on effort-related choice behavior. After a seven day recovery period, rats were retrained on their respective behavioral tasks to reestablish their baseline responding rates. Pumps were pre-programmed to initiate an infusion rate of 3 µl/hour twelve days after surgery. As soon as animals reached stable baseline responding, pumps were filled with haloperidol (2.0 mg/ml or 1.0 mg/ml) or 0.3% tartaric acid vehicle through a subcutaneous port. Infusion of haloperidol or vehicle occurred for four weeks, which was referred to as the 'drug exposure' phase, followed by a four-week 'washout phase'. During both the drug exposure and washout phases, behavioral testing was conducted 5 days/week (19 days of data collection in each phase).

*Statistical Analysis*

The data for this experiment were analyzed in SPSS using a 3 × 4 factorial analysis of variance (ANOVA) with treatment group as the between-groups factor and week as the within-groups factor. In the case of a significant interaction, simple effects of treatment group were assessed by computing between groups ANOVAs at each week. Dunnett's tests using the error term from the between-subjects analysis were used to assess differences between each drug treatment and vehicle at each time point (weeks 1-4). A p value of $<0.05$ was considered statistically significant for all analyses.

## 2.3 Results

***Experiment 2.1:*** *Ability of chronic DA $D_2$ receptor antagonism by continuous subdermal infusion of haloperidol to suppress lever pressing on the FR5 operant procedure.*

Fifteen rats were treated with one of two doses of haloperidol (1.0 mg/ml or 2.0 mg/ml) or vehicle for four weeks and tested on the FR5 lever pressing task daily. At baseline, prior to drug exposure, there were no significant differences in lever pressing between the three groups according to one-way ANOVA (p=n.s.). During the drug exposure phase, repeated measures factorial ANOVA indicated a significant overall effect of treatment group [$F(2,12)=36.172$, $p<0.001$] and week [$F(3,36)=3.434$, $p<0.05$] on lever pressing **(Figure 2.1)**. No week by treatment interaction was found (p=n.s.), but a linear trend for week approached significance [$F(1,12)=3.617$, $p=0.08$]. To make specific comparisons of the effects of each dose of haloperidol and assess effect size of treatments on lever pressing, factorial ANOVAs were performed comparing the vehicle vs. high dose group, and vehicle vs. low dose group. Treatment had a significant effect on high dose group lever pressing [$F(1,9)=150.137$, $p<0.001$] with a large effect size ($\eta p^2 = 0.943$). The low dose treatment did not have a significant effect on lever presses [$F(1,8)=4.739$, $p>0.05$] and the effect size for this analysis was relatively low ($\eta p^2 = 0.372$).

In the washout phase, factorial ANOVA revealed a significant effect of week [$F(3,36)=18.255$, $p<0.001$] and a significant week by treatment interaction [$F(6,36)=6.073$, $p<0.001$], and no significant overall effect of treatment on lever pressing during the washout phase (p=n.s.). Because of a significant week by treatment interaction, between groups overall ANOVAs were computed at each time point and post-hoc two-tailed Dunnett's tests were performed to assess differences between individual groups. An overall significant difference in

lever presses was found only at week 1 [$F(2,14)=4.297$, $p<0.05$], and a Dunnett's test revealed a significant difference only between the vehicle and high dose groups ($p<0.05$). No significant differences in lever pressing were seen in the remaining weeks of washout. In addition, a significant linear trend for week [$F(1,12)=40.368$, $p<0.001$], and for week by treatment interaction [$F(2,12)=11.957$, $p<0.001$] were found during washout. Thus, in summary, the high dose of haloperidol suppressed FR5 lever pressing, the lack of significant dose x treatment interaction indicates that there were no substantial signs of sensitization or tolerance during chronic drug treatment, and there was rapid return to pre-drug lever pressing during washout (**Fig. 2.1**).

***Experiment 2.2:*** *Ability of chronic DA $D_2$ receptor antagonism by continuous subdermal infusion of haloperidol to induce motivational impairments on the FR5/chow feeding choice procedure.*

**Figure 2.2** shows the effects of chronic haloperidol treatment on lever pressing and chow intake on the FR5/chow feeding choice task in rats (n=17). During the drug exposure phase, there was a significant overall effect of treatment on lever presses [$F(2,14)=20.498$, $p<0.001$] (**Fig. 2.2A**) and chow intake [$F(2,14)=18.574$, $p<0.001$] (**Fig. 2.2B**), but no effect of week or week by treatment interaction for either lever presses or chow intake (p=n.s.). As with Exp 2.1, separate factorial ANOVAs were computed to assess the effect of treatment on lever pressing in the vehicle vs. high dose group and vehicle vs. low dose group. Significant effects of treatment on lever presses were found when comparing vehicle vs. high dose [$F(1,9)=39.845$, $p<0.001$; $\eta p^2 = 0.816$], and vehicle vs. low dose [$F(1,7)=20.359$, $p<0.01$; $\eta p^2 = 0.744$], with large effect sizes between drug treatment and lever presses for both doses.

The total number of subjects was reduced to n=7 in the washout phase. Repeated measures ANOVA revealed a significant effect of week on lever presses [$F(3,12)=11.088$, $p=0.001$] and chow intake [$F(3,12)=8.813$, $p<0.01$]. The week by treatment interaction approached significance for lever presses ($0.05<p<0.1$), and was statistically significant for chow intake [$F(6,12)=3.194$, $p<0.05$]. Treatment did not have a significant overall main effect on lever presses or chow intake during the washout phase (p=n.s.).

In summary, both the low and high doses of haloperidol shifted choice behavior, suppressing FR5 lever pressing while significantly and substantially increasing chow intake. As seen in the FR5 alone experiment, there was a lack of significant dose x treatment interaction during chronic drug treatment, indicating that there were no substantial signs of sensitization or tolerance. Although the n was very low during the washout period due to the COVID shutdown, it is evident that there was a rapid return to pre-baseline levels of lever pressing during the washout, though the chow data were more variable (**Fig. 2.2**).

## 2.4 Figures

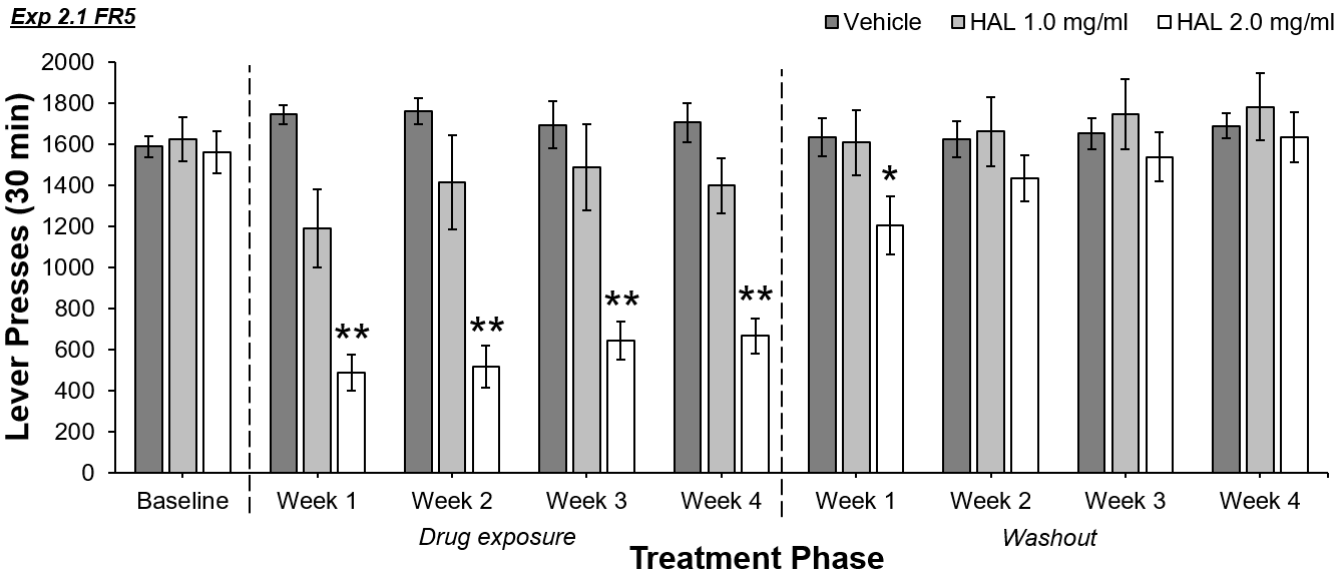



**Figure 2.1.** The effect of chronic haloperidol (HAL) administration on lever pressing on an FR5 feeding task in rats (n=15). Mean (± SEM) number of lever presses during a 30-minute session. **p<0.001, significant between groups effect of treatment on lever presses between HAL 2.0 mg/ml vehicle during drug exposure phase. *p<0.05, lever presses significantly differed between HAL 2.0 mg/ml and vehicle at week 1 of washout. Dashed vertical lines represent treatment phase transitions.

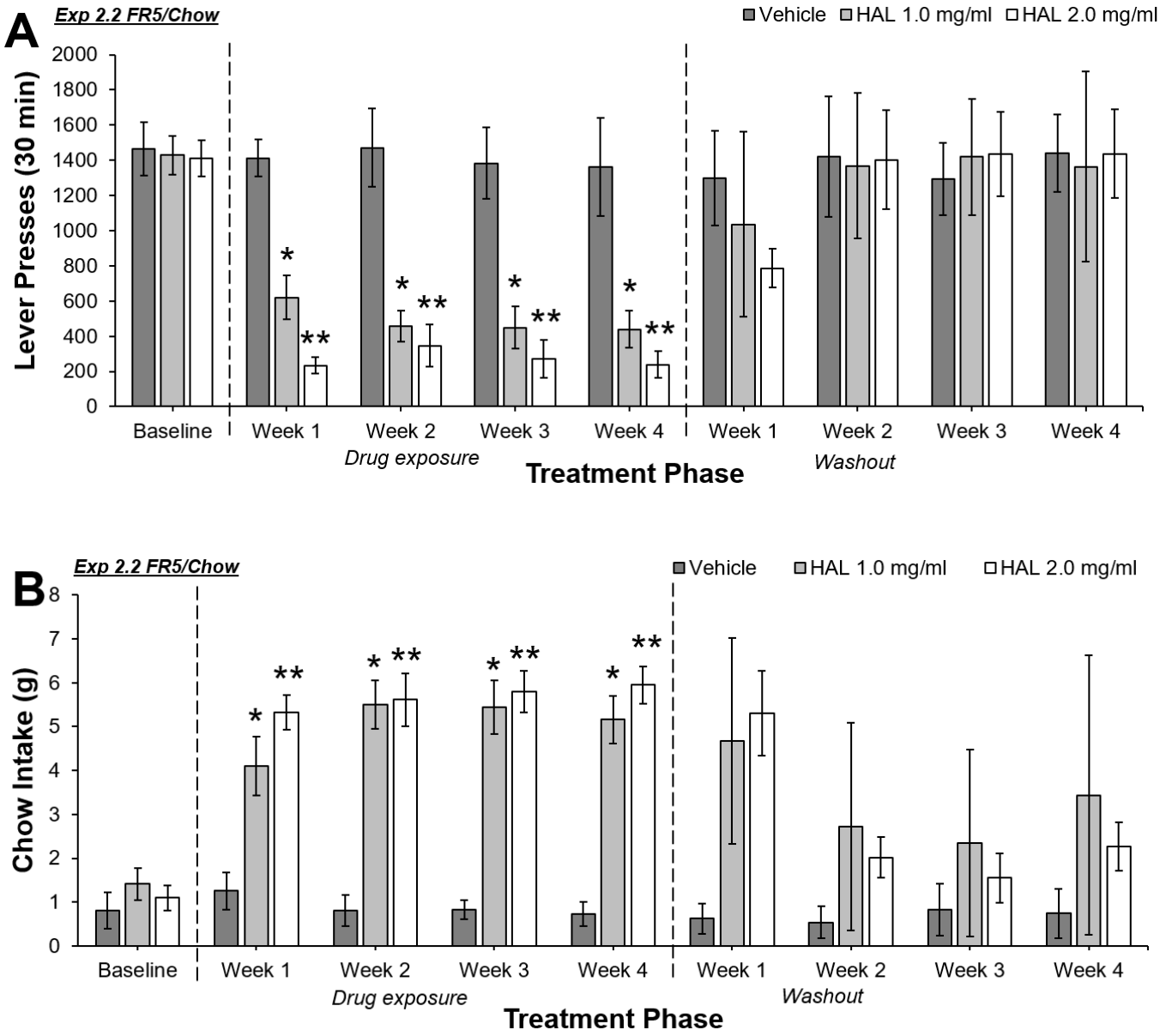



**Figure 2.2.** The effect of chronic haloperidol (HAL) administration on lever pressing and chow intake in rats (n=17) performing on an FR5/chow feeding choice task. **(A)** Mean (± SEM) number of lever presses in a 30-minute session. **p<0.001, significant between groups effect of treatment on lever presses between HAL 2.0 mg/ml and vehicle, *p<0.01 significant between groups effect of treatment on lever presses between HAL 1.0 mg/ml and vehicle during drug exposure phase. **(B)** Mean (± SEM) gram quantity of chow intake during a 30-minute session.

**p<0.001, significant between groups effect of treatment on chow intake between HAL 2.0 mg/ml and vehicle, *p<0.01 significant between groups effect of treatment on chow intake between HAL 1.0 mg/ml and vehicle during drug exposure phase. There was a significant effect of week (p<0.01) and a significant week by treatment interaction (p<0.05) only during the washout phase. Dashed vertical lines represent treatment phase transitions.

**Chapter 3: The novel atypical dopamine uptake inhibitor *(S)*-CE-123 partially reverses the effort-related effects of the dopamine depleting agent tetrabenazine and increases progressive ratio responding.**

Published; Rotolo et al. 2019, *Frontiers in Pharmacology*

## 3.1 Introduction

Motivational symptoms such as fatigue, anergia, and psychomotor slowing are seen in depression, Parkinson's disease, and other disorders. These symptoms are often debilitating, and can severely limit long-term functional outcomes (Demyttenaere et al. 2005; Friedman et al. 2007; Fava et al. 2014; Rothschild et al. 2014; Chong et al. 2015; Salamone et al. 2006, 2016a,b,c, 2017). Moreover, motivational symptoms can be highly resistant to treatment with common antidepressants such as serotonin transporter (SERT) blockers (Cooper et al. 2014; Fava et al. 2014; Rothschild et al. 2014). The catecholamine uptake inhibitor bupropion has shown some success in treating motivational symptoms in depressed people (Papakostas et al. 2006; Pae et al. 2006; Cooper et al. 2014), and reports indicate that treatment with drugs that inhibit dopamine transporter (DAT), such as d-amphetamine and methylphenidate, can improve motivational function (Stotz et al. 1999). However, psychomotor stimulants that block DAT also can have undesirable effects, such as abuse liability and induction of psychotic symptoms. For these reasons, it is important to develop and assess drugs that are highly selective for DAT, but show atypical neurochemical characteristics that may lower the side effect profile.

A recently synthesized and chromatographically separated analog of modafinil, ***(S)***-CE-123 (**Fig. 3.1**) is a highly selective atypical inhibitor of DAT which has been shown to enhance

cognitive flexibility and reduce impulsivity in rats (Nikiforuk et al., 2017). The present paper describes the enantioselective synthesis and initial characterization of ***(S)***-CE-123. In addition, ***(S)***-CE-123 was assessed for its effects on effort-related aspects of motivation in rats. These behavioral pharmacology studies evaluated the motivational effects of ***(S)***-CE-123 in rats using well characterized tests of effort-based choice behavior, the concurrent fixed ratio 5 (FR5)/chow feeding choice task, and the concurrent progressive ratio (PROG)/chow feeding choice task. Effort-related choice is studied using procedures that offer high effort options leading to highly valued reinforcers vs. low effort/low reward options (Salamone and Correa 2012; Salamone et al. 2016a,b,c, 2017, 2018; Hart et al. 2017). Considerable research has implicated dopamine (DA) transmission, particularly in nucleus accumbens core, in the regulation of effort-based choice; animals with impaired DA transmission (i.e., DA antagonism or depletion) show a shift from the high-effort option to the low effort option, while enhancement of DA transmission reverses those effects (Sokolowski and Salamone 1998; Salamone et al. 2002, 2007, 2016a,b,c; Farrar et al. 2010; Mai et al. 2012; Hosking et al. 2015). It has been suggested that animal models employing tests of effort-based decision making can be used to study functions that are related to aspects of human motivational dysfunction (Salamone et al. 2006, 2016a,b,c, 2018). This strategy has been validated by clinical research showing that patients with major depression, Parkinson's disease, and other disorders show a low-effort bias when tested on effort-related choice procedures (Treadway et al. 2012; Yang et al. 2014; Chong et al. 2015; Barch et al. 2017).

Studies have shown that a low-effort bias can be induced in rats by several conditions associated with depressive symptoms, including stress (Shafiei et al. 2012; Bryce and Floresco 2016), inflammatory challenge (Nunes et al. 2014; Yohn et al. 2016e), withdrawal from methamphetamine (Hart et al. 2018), and injections of the vesicular monoamine transporter type-

2 (VMAT-2) inhibitor tetrabenazine (TBZ; Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2015a,b). TBZ induces depressive symptoms in people (Frank 2009, 2010; Guay, 2010), and has been used in classical animal models of depression (Tadano et al., 2000; Wang et al., 2010). By virtue of inhibiting VMAT-2, TBZ blocks DA storage, depletes brain DA, and reduces post-synaptic DA receptor signaling (Nunes et al. 2013). Across a variety of behavioral tests, TBZ shifts choice behavior and induces a low-effort bias, and the reallocation of behavior from high to low-effort options that is produced by TBZ is not due to alterations in food preference or hedonic taste reactivity, reduced appetite, or impairments in reference memory (Randall et al. 2012, 2014; Nunes et al. 2013; Correa et al. 2015; Pardo et al. 2015; Yohn et al. 2015a). On operant behavior tests that give animals the choice between lever pressing on a FR5 or PROG schedule for a preferred food vs. approaching and consuming a less preferred lab chow (Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2016a,b,c) TBZ reliably decreases working for food by lever pressing, but actually increases consumption of the concurrently available chow. TBZ in this dose range does not reduce consumption of either type of food that is used in these studies, nor does it affect preference as measured in free-feeding preference tests (Nunes et al. 2013). Also, the effects of TBZ on operant choice procedures differ substantially from the effects of reinforcer devaluation, as well as appetite suppressant drugs (Randall et al. 2013, 2014). These effects of TBZ are not reversed by the SERT blockers fluoxetine and citalopram, or by the norepinephrine transporter (NET) inhibitor desipramine (Yohn et al. 2016a,b), but they are attenuated by several drugs that block DAT, including bupropion, GBR12909, lisdexamfetamine, methylphenidate and modafinil (Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2016a,b,c; Salamone et al. 2016a,b). Drugs that inhibit DAT, such as bupropion, lisdexamfetamine, and PRX-14040, also have been shown to increase the motivation to work for food reinforcers in

more highly demanding behavioral tasks, such as the PROG/chow feeding choice task (Randall et al. 2015; Yohn et al. 2016b,c,d). In view of these results, the present studies investigated the ability of ***(S)***-CE-123 to attenuate the effort-related effects of TBZ in rats tested on the concurrent FR5/chow feeding choice task, and to enhance exertion of effort in rats assessed with the PROG/chow feeding choice task. An additional experiment studied the effects of the behaviorally effective dose of ***(S)***-CE-123 (24.0 mg/kg) on extracellular DA in nucleus accumbens using microdialysis methods.

## 3.2 Materials and Methods

*Synthesis of (S)-CE-123*

Refer to Rotolo et al. 2019 for details regarding synthesis and chemical structure (**see Fig. 3.1**). Synthesis was performed by the laboratory of Dr. Gert Lubec.

*In Vivo Behavioral Pharmacology*

*Animals:*

For the behavioral pharmacology experiments, adult male Sprague Dawley rats (Envigo Sprague Dawley, Indianapolis, IN, USA; weights 275-299 g upon arrival) were pair-housed in a colony maintained at 23°C, with a 12-h light/dark cycle (lights on 07:00). Rats were food deprived to 85% of their free-feeding body weight for operant training and allowed modest growth throughout the experiment. Water was available ad libitum in the home cages. Animal protocols were approved by the University of Connecticut Institutional Animal Care and Use Committee, and the studies were conducted according to National Institutes of Health (NIH) guidelines. For the *in vivo* microdialysis experiment, adult male Sprague Dawley rats (Harlan

Italy; weights 275-300 g upon arrival) were used. Rats were housed 4 per cage, in standard plastic cages with wood chip bedding, maintained at 22 ± 2 °C and 60% humidity with a 12-h light/dark cycle (lights on 07:00). Water and standard laboratory rodent chow (Mucedola, Settimo Milanese, Italy) were provided ad libitum in the home cage. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.L. 116/92 and 152/06) and European Council (609/86 and 63/2010) directives and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health (Aut. N. 162/2016- PR). All efforts were taken to minimize pain and suffering, and to reduce the number of animals used.

*Behavioral Procedures*

*FR5/Chow Feding Choice Task:*

Behavioral sessions were conducted in operant chambers (28 x 23 x 23 $cm^3$; Med Associates, Fairfax, VT) with 30 minute sessions 5 days/week. Rats were initially trained to lever press on a continuous reinforcement FR1 schedule (high-carbohydrate 45 mg pellets, Bio-Serv, Frenchtown, NJ) and then shifted to the FR5 schedule. After 5 weeks of training on the FR5 schedule, chow was introduced. Weighed amounts of laboratory chow (Laboratory Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO; typically 15-20 g) were concurrently available on the floor of the chamber during the FR5/chow feeding choice task sessions. At the start of each session, it was confirmed that the pieces of weighed chow were larger than the spaces between the bars that make up the floor of the chamber, so they could not fall through. At the end of each 30 minute session, rats were immediately removed from the chambers, number

of lever presses was recorded, and the amount of chow consumed was determined by weighing the remaining food (including spillage from a tray beneath the floor of the chamber). Rats were trained on the FR5/chow feeding choice procedure for 5 weeks, after which drug testing began. On baseline and drug treatment days, rats consumed all of the operant pellets that were delivered during each session.

*PROG/Chow Feeding Choice Task:*

Behavioral sessions were conducted in operant chambers with 30 minute sessions 5 days/week. Rats were initially trained to lever press on a continuous reinforcement FR1 schedule (high-carbohydrate 45 mg pellets, Bio-Serv) and then shifted to the PROG schedule (Randall et al. 2012, 2014, 2015). For PROG sessions, the ratio started at FR1 and was increased by 1 additional response every time 15 reinforcements were obtained (FR1×15, FR2×15, etc.). A "time-out" feature deactivated the response lever for the rest of the session whenever 2 minutes elapsed without a completed ratio. After 9 weeks of training on the PROG schedule, chow was introduced. Weighed amounts of laboratory chow (Laboratory Diet, 5P00 Prolab RMH 3000, Purina Mills; typically 15-20 g) were concurrently available on the floor of the chamber during the PROG/chow feeding choice task sessions. At the end of each 30 minute session, rats were immediately removed from the chambers, number of lever presses was recorded, and the amount of chow consumed was determined by weighing the remaining food (including spillage from a tray beneath the floor of the chamber). Rats were trained on the PROG/chow feeding choice procedure for 5 weeks, after which drug testing began. On baseline and drug treatment days, rats consumed all of the operant pellets that were delivered during each session.

*Drug Treatments and Dose Selection*

***(S)***-CE-123 (***(S)***-5-((benzhydrylsulfinyl)methyl)thiazole) was obtained from the Lubec Laboratory (University of Vienna, Austria) and dissolved in dimethyl sulfoxide (DMSO) (10%), Tween 80 (15%), and 0.9% saline (75%). The DMSO/Tween 80/saline solution was administered as the vehicle control. TBZ (9,10-dimethoxy-3-(2-methylpropyl)-1,3,4,6,7, 11b hexahydrobenzo[a]quinolizin-2-one) was obtained from Tocris Bioscience (Ellisville, MO) and was dissolved in DMSO (20%), 0.9% saline (80%), and was titrated with microliter quantities of 1.0 N HCl until the solid drug was in solution at a pH of 4.0-4.5. The DMSO/saline solution was administered as the vehicle control. The dose of 1.0 mg/kg TBZ was based on extensive piloting in our laboratory. The doses of ***(S)***-CE-123 were selected based on extensive pilot studies and information about its relative affinity for DAT.

*Behavioral Pharmacology Experiments*

Trained rats (n=8) were administered either TBZ (1.0 mg/kg) or vehicle, and ***(S)***-CE-123 (6.0, 12.0, and 24.0 mg/kg) or vehicle, via intraperitoneal (IP) injections on drug testing days. Rats received TBZ or vehicle 120 minutes before testing and ***(S)***-CE-123 or vehicle 30 minutes before testing. The experiment used a within-groups design, with each rat receiving each drug treatment in a randomly varied order (one treatment per week, with none of the treatment sequences repeated across different animals). Sample size was determined based on information obtained from pilot studies done in our laboratory. Experimenter blinding was not necessary due to the objective nature of the data collection. The following five treatment combinations were given: TBZ vehicle + ***(S)***-CE-123 vehicle; 1.0 mg/kg TBZ + ***(S)***-CE-123 vehicle; 1/0 mg/kg TBZ + 6.0 mg/kg ***(S)***-CE-123; 1.0 mg/kg TBZ + 12.0 mg/kg ***(S)***-CE-123; 1.0 mg/kg TBZ + 24.0

mg/kg ***(S)***-CE-123. The endpoint of the experiment was marked by the completion of the last scheduled drug treatments for each rat, after having received each drug treatment in a randomly varied order.

Following the initial behavioral experiment, ***(S)***-CE-123 was tested on a different group of rats (n=7) to assess whether it had a behavioral effect when administered alone. Rats were trained on the FR5/chow feeding choice task as described previously. Thirty minutes prior to the testing session, rats were administered either vehicle or 24.0 mg/kg ***(S)***-CE-123. One week later, rats received either vehicle or 24.0 mg/kg ***(S)***-CE-123 so that the treatment orders were counterbalanced. Only the highest dose of ***(S)***-CE-123 (24.0 mg/kg) was chosen for testing in this paradigm.

A third behavioral experiment was conducted to determine if ***(S)***-CE-123 had an effect on rats' behavior on the PROG/chow feeding choice task when administered alone. Thirty minutes prior to the testing session, trained rats (n=15) were administered either vehicle or 6.0, 12.0, or 24.0 mg/kg ***(S)***-CE-123. This experiment used a within-groups design, with each rat receiving each drug treatment in a randomly varied order. Treatments were administered once per week over the course of four weeks.

*In Vivo Microdialysis*

*Surgery:*

Male Sprague Dawley rats (275-300 g; Harlan, Italy) were anaesthetized with fentanyl (0.06 mg/kg IP), placed in a stereotaxic apparatus, and implanted with vertical dialysis probes prepared as previously described (De Luca et al., 2016) with 1.5 mm dialyzing portion. According to the rat brain atlas of Paxinos and Watson (1998), unilateral probes were implanted

in the nucleus accumbens shell (A +2.2, L +1.0 from bregma, V -7.8 from dura; n=10) or nucleus accumbens core (A +1.4; L +1.6 from bregma, V -7.6 from dura; n=7).

*Analytical Procedure:*

On the day following surgery, animals were connected to an infusion pump and probes were perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$) at a constant rate of 1 µl/min. After rinsing for at least 1 h, dialysate samples (20 µl) were collected every 20 minutes and injected into an HPLC equipped with a reverse phase column (C8 3.5 µm, Waters, USA) and a coulometric detector (ESA, Coulochem II) to quantify DA. The electrodes of the analytical cell were set at +125 mV (oxidation) and −175 mV (reduction) to detect dopamine. The mobile phase composition was: 50 mM $NaH_2PO_4$, 0.1mM $Na_2EDTA$, 0.5 mM n-octylsulfate and 15% (v/v) methanol. The sensitivity of the assay for DA was 5 fmol/sample. When the DA did not differ more than 10% in three consecutive samples, the average value was considered as the basal level of DA. The animals were treated with vehicle or ***(S)***-CE-123 24.0 mg/kg IP, and monoamine levels were monitored for 3 h from the start of the treatment.

*Histology:*

At the end of the experiment, animals were sacrificed and brains were removed and stored in formalin (8%). Brains were sliced and stained with Nissl stain for histological examination in order to verify the correct placement of the microdialysis probes. Histology work was performed by the laboratory of Dr. Marco Pistis.

*Statistical Analysis*

*Behavioral Pharmacology Experiments:*

Total number of lever presses and gram quantity of chow intake from the 30 minute sessions were analyzed using repeated measures ANOVA. A statistical program (SPSS, Version 25) was used to perform all analyses. Since there were significant overall F values for the three behavioral measures being used, nonorthogonal planned comparisons were performed, using the overall error term to assess differences between each treatment and the control condition. The number of comparisons was restricted to the number of treatments minus one (Keppel, 1991). Statistical outliers were predefined as any point that is more than two standard deviations from the mean. No data from this study were excluded as outliers. Microdialysis data were expressed as percent of the last 3 baseline samples, and were analyzed by a 4 group x 12 sample factorial analysis of variance (ANOVA) with repeated measures on the sample factor. Since there was a significant group x sample interaction, so subsequent analysis of simple main effects examined the effect of the sample factor for each of the 4 groups. Results from treatments showing significant overall changes were subjected to Tukey's tests for post hoc comparisons, with significance at $p<0.05$.

## 3.3 Results

*FR5/Chow Feeding Choice task*

**Figure 3.2** shows the results of the FR5/chow feeding choice experiment. TBZ shifted effort-based choice, decreasing lever pressing and increasing chow intake. Repeated measures ANOVA revealed that there was an overall significant effect of drug treatment on lever pressing [$F(4,28)=34.625$, $p<0.001$] (**Fig. 3.2A**). Planned comparisons showed that TBZ significantly

decreased lever pressing compared to vehicle treatment (TBZ/Veh vs. Veh/Veh [$F(1,28) = 105.87$, $p<0.001$]). There also was a significant overall effects of drug treatment on chow intake [$F(4,28)=27.280$, $p<0.001$], and TBZ alone significantly increased chow intake relative to vehicle treatment (TBZ/Veh vs. Veh/Veh [$F(1,28) = 66.625$, $p<0.001$]) (**Fig. 3.2B**). Additional planned comparisons revealed that co-administration of the dose of 24.0 mg/kg ***(S)***-CE-123 with TBZ significantly attenuated the effects of TBZ on lever pressing (TBZ plus 24.0 mg/kg vs. TBZ/Veh [$F(1,28) = 13.2866$, $p<0.01$]) and chow intake (TBZ plus 24.0 mg/kg vs. TBZ/Veh [$F(1,28) = 61.014$, $p<0.001$]).

As shown in Figure 3, there was no effect of drug treatment relative to vehicle on lever pressing or chow intake on the FR5/chow feeding choice task when 24.0 mg/kg ***(S)***-CE-123 was administered alone prior to testing. Repeated measures ANOVA indicated no significant difference between rats treated with 24.0 mg/kg ***(S)***-CE-123 and vehicle-treated rats on lever pressing [$F(1,6)=0.012$, p=n.s.] (**Fig. 3.3A**) or chow intake [$F(1,6)=0.030$, p=n.s.] (**Fig. 3.3B**).

*PROG/Chow Feeding Choice Task*

***(S)***-CE-123 was tested for its behavioral effect on the PROG/chow feeding choice task when administered alone (i.e., in the absence of TBZ). Repeated measures ANOVA revealed an overall main effect of drug treatment on lever pressing [$F(3,42)=3.165$, $p<0.05$], and planned comparisons demonstrated that lever presses were significantly increased at 24.0 mg/kg ***(S)***-CE-123 compared to vehicle treatment (Veh vs. 24.0 mg/kg ***(S)***-CE-123 [$F(1,42)=4.061$, $p<0.05$] (**Fig. 4.4A**). Drug treatment with ***(S)***-CE-123 also had a significant effect on chow intake during the PROG/chow session, indicated by a repeated measures ANOVA [$F(3,42)=13.771$, $p<0.001$],

with a significant reduction in chow intake at the 24.0 mg/kg dose compared to vehicle (Veh vs. 24.0 mg/kg ***(S)***-CE-123 [$F(1,42)=28.107$, $p<0.001$] (**Fig. 3.4B**).

*Microdialysis*

One-way ANOVA revealed no differences in basal DA levels between nucleus accumbens shell vehicle (Mean 130.46 fmoles DA/20 µl; SEM 19.01) and nucleus accumbens shell 24.0 mg/kg ***(S)***-CE-123 (Mean 90.65 fmoles DA/20 µl; SEM 12.17) conditions [$F(1,9)=2.411$, p=n.s.], or nucleus accumbens core vehicle (Mean 91.28 fmoles DA/20 µl; SEM 27.37) and nucleus accumbens core 24.0 mg/kg ***(S)***-CE-123 (Mean 90.25 fmoles DA/20 µl; SEM 17.12) conditions [$F(1,6)=0.001$, p=n.s.]. A repeated measures factorial ANOVA revealed a significant overall effect of treatment group [$F(3,13)=29.680$, $p<0.001$], a significant effect of sample [$F(11,143)=6.756$, $p<0.001$], and a significant treatment x sample interaction [$F(33,143)=8.019$, $p<0.001$] (**Fig. 3.5**). Analysis of simple effects in which each condition was analyzed separately showed that there was a significant increase in extracellular DA in the nucleus accumbens core in the group that received 24.0 mg/kg ***(S)***-CE-123 [$F(11,33)=11.950$, $p<0.001$]. Tukey tests indicated that 24.0 mg/kg ***(S)***-CE-123 administration increased nucleus accumbens core DA significantly from baseline at samples taken 40-180 minutes after injection ($p<0.05$). Analysis of simple effects also showed that there was a significant difference in extracellular DA in the nucleus accumbens shell in the group treated with 24.0 mg/kg ***(S)***-CE-123 [$F(11,33)=7.049$, $p<0.001$] from baseline, and a Tukey test indicated a significant reduction at 100 minutes after injection ($p<0.05$). Injections of vehicle had no significant effect on extracellular DA levels in either nucleus accumbens core or shell (**Fig. 3.5**).

### 3.4 Figures

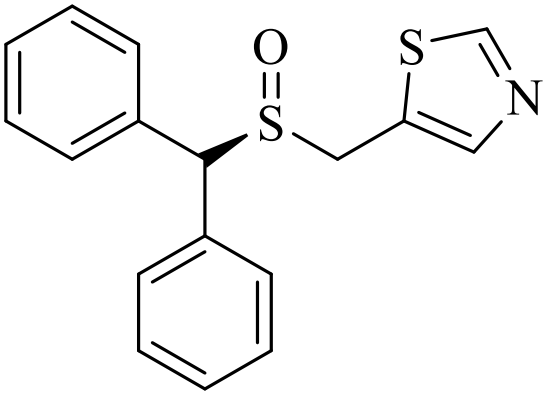
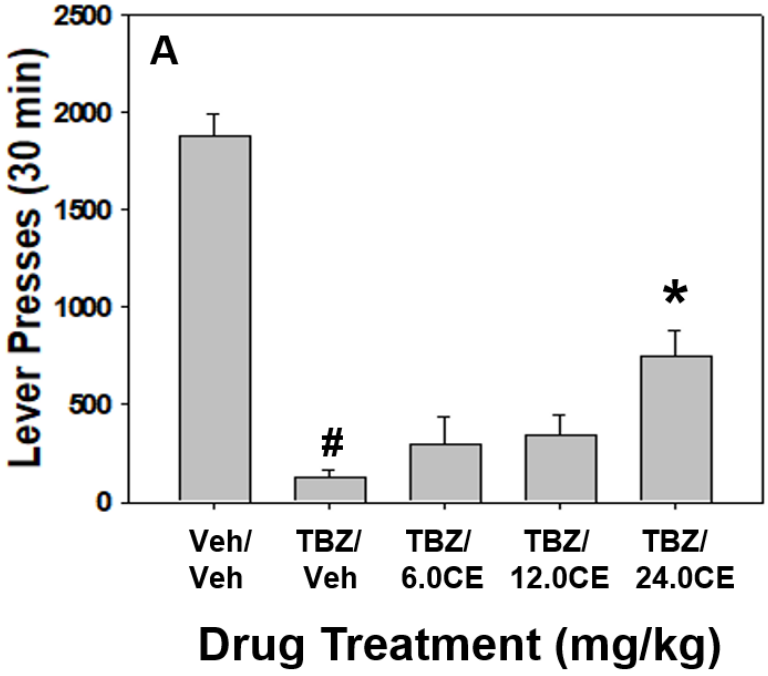



**Figure 3.1.** Structure of the novel atypical dopamine reuptake inhibitor (S)-CE-123 ((S)-5-((benzhydrylsulfinyl)methyl)thiazole).



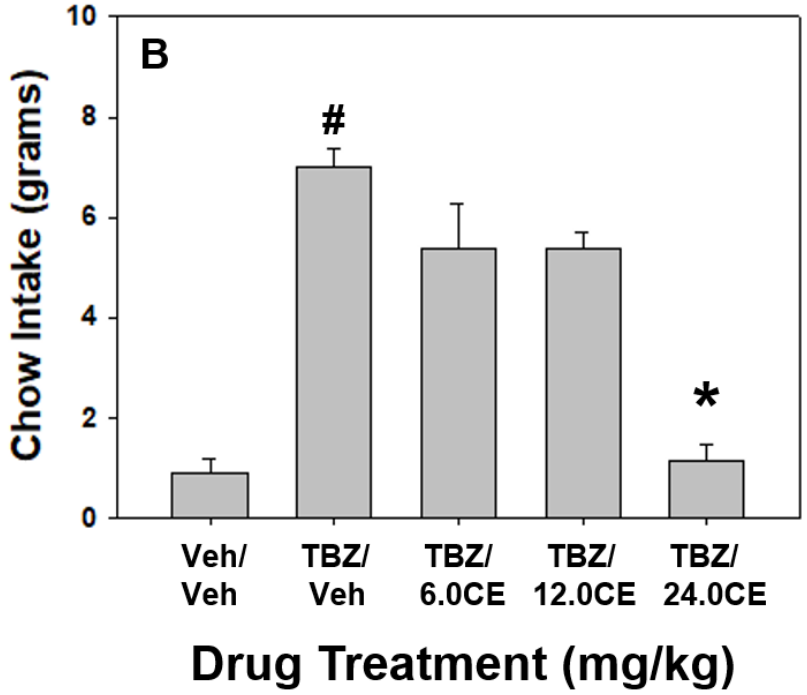



**Figure 3.2.** The effects of the DAT blocker ***(S)***-CE-123 on TBZ-induced changes in performance on the concurrent lever pressing/chow-feeding choice procedure. **(A)** TBZ plus vehicle significantly differed from vehicle plus vehicle (#$p$<0.001); TBZ plus 24.0 mg/kg ***(S)***-CE-123 significantly differed from TBZ plus vehicle (*$p$< 0.01). **(B)** TBZ plus vehicle significantly differed from vehicle plus vehicle (#$p$<0.001); TBZ plus 24.0 mg/kg ***(S)***-CE-123 significantly differed from TBZ plus vehicle (**$p$< 0.001).

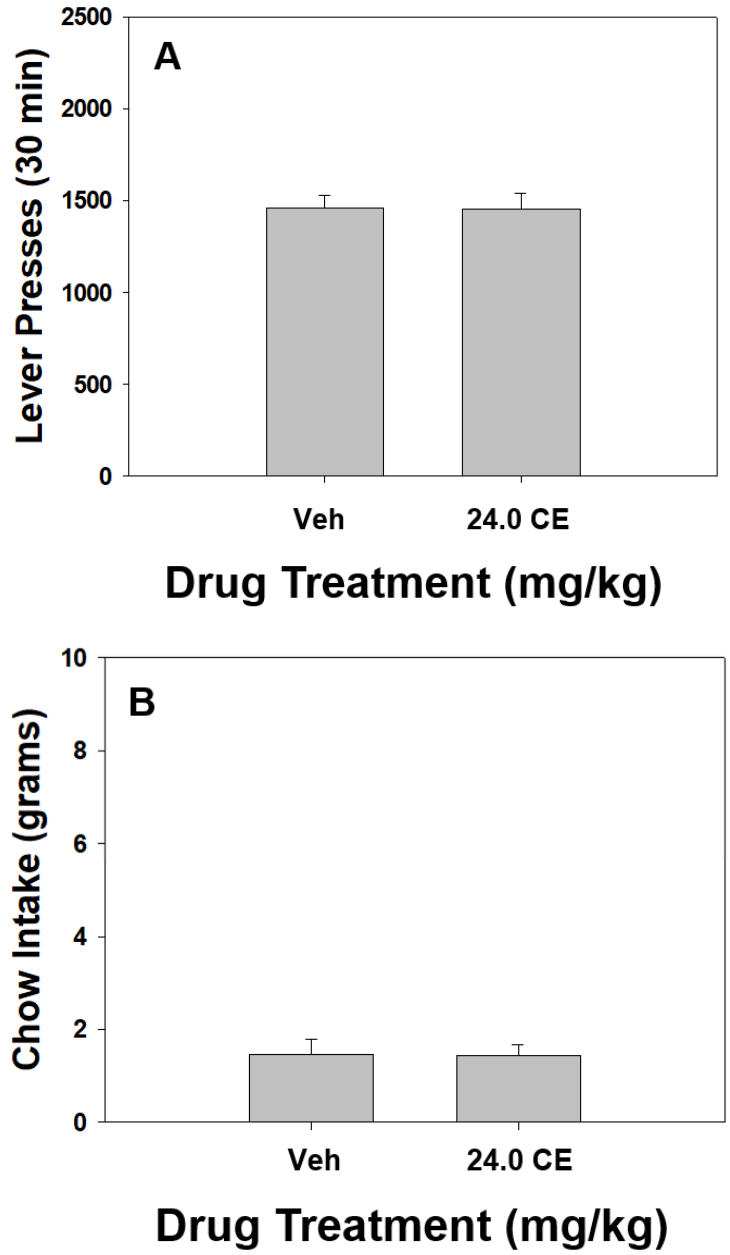



**Figure 3.3.** The effects of 24.0 mg/kg ***(S)***-CE-123 or vehicle on performance on the concurrent FR5/chow feeding choice task. There was no effect of drug treatment on lever pressing or chow intake on the FR5/chow feeding choice task when rats (n=7) were administered ***(S)***-CE-123 or vehicle prior to testing. Repeated measures ANOVA indicated no significant difference between

rats treated with 24 mg/kg ***(S)***-CE-123 and vehicle-treated rats on lever pressing [F(1,6)=0.012, p=n.s.] (**Fig. 3.3A**) or chow intake [F(1,6)=0.030, p=n.s.] (**Fig. 3.3B**).

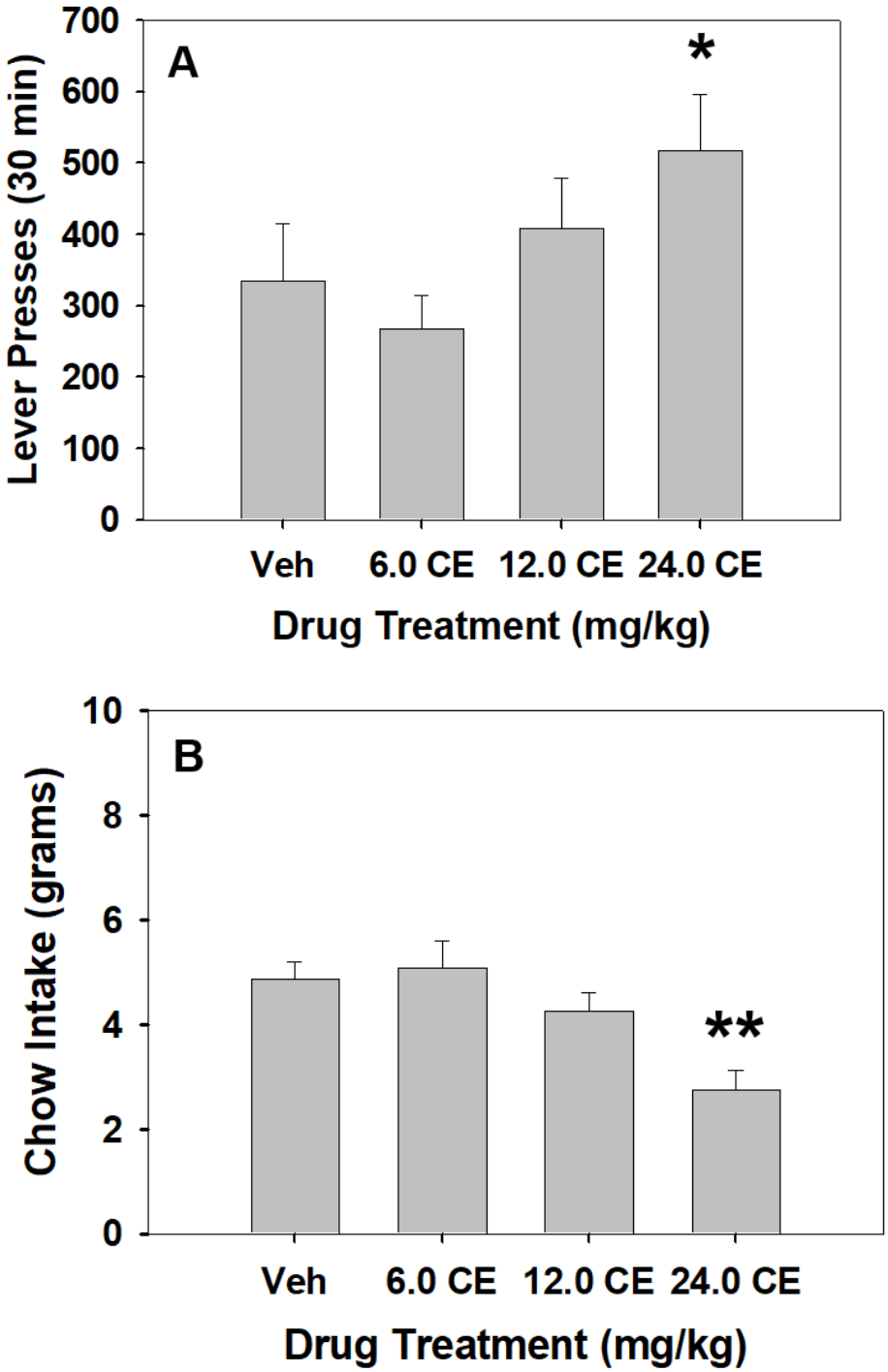


**Figure 3.4.** The effects of the DAT blocker ***(S)***-CE-123 on performance on the concurrent PROG/chow feeding choice procedure. Rats (n=15) received intraperitoneal injections of vehicle (Veh), 6.0, 12.0, or 24.0 mg/kg doses of (*S*)-CE-123. **(A)** 24.0 mg/kg (*S*)-CE-123 significantly differed from vehicle (**p*<0.05). **(B)** 24.0 mg/kg (*S*)-CE-123 significantly differed from vehicle (***p*<0.001)

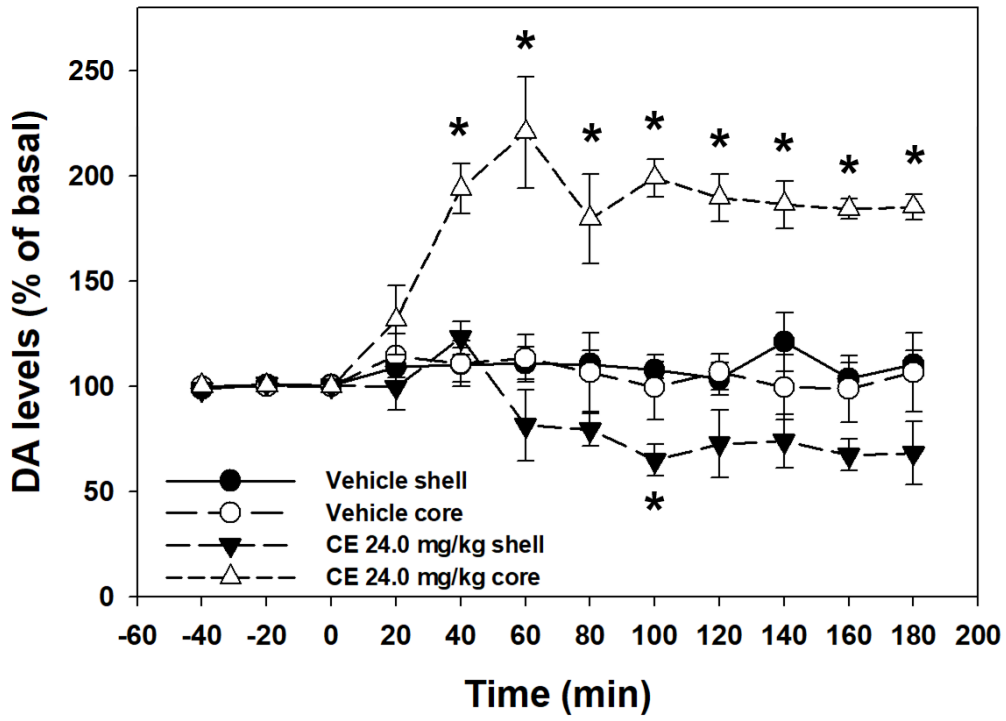



**Figure 3.5.** Effect of vehicle or 24.0 mg/kg ***(S)***-CE-123 on extracellular DA in the nucleus accumbens core and shell measured by microdialysis. Mean (±SEM) extracellular DA (expressed as percent baseline) in 20 minute samples across 180 minutes. Three baseline samples were collected prior to injection, followed by nine post-injection samples. *significantly different from vehicle, p<0.05.

**Chapter 4: Behavioral and dopamine transporter binding properties of the modafinil analog *(S, S)*-CE-158: reversal of the motivational effects of tetrabenazine and enhancement of progressive ratio responding.**

Submitted; Rotolo et al., *Psychopharmacology*

## 4.1 Introduction

Motivational symptoms such as anergia, fatigue, and effort-related dysfunctions are common and debilitating features of major depressive disorder, schizophrenia, Parkinson's disease and other psychiatric and neurological disorders (Tylee et al. 1999; Demyttenaere et al. 2005; Treadway et al. 2012; Barch et al. 2014; Chong et al. 2015). Motivational symptoms are not well treated by many of the most commonly prescribed antidepressant drugs (Tylee et al. 1999; Demyttenaere et al. 2005; Cooper et al. 2014; Fava et al. 2014; Ghanean et al. 2018). Patients treated with drugs that block the serotonin transporter (SERT), such as fluoxetine, sertraline, paroxetine or escitalopram, often suffer from residual symptoms of apathy, fatigue, and a lack of energy (Katz et al. 2004; Padala et al. 2012; Rothschild et al. 2013; Cooper et al. 2014; Ferguson et al. 2014), which can be detrimental to daily functioning. Clinical reports have revealed a critical link between symptoms related to fatigue and energy expenditure and the overall severity of depressive symptoms (Gullion and Rush 1998; Fava et al. 2014; Chung et al. 2015; Ghanean et al. 2018). Moreover, self-reported fatigue is correlated with global functioning deficits and reduced likelihood of remission (Ferguson et al. 2014). The array and prevalence of symptoms left unabated by antidepressant medications emphasizes the importance of utilizing

animal models of fatigue and anergia to aid in the development of new and more effective treatments.

In order to conduct preclinical studies of motivational dysfunctions using animal models, tasks have been developed to evaluate effort-based decision making in rodents (Salamone et al. 2006, 2007, 2016a,b, 2018; Mai et al. 2012; Nunes et al. 2013; Sommer et al. 2014; Winstanley and Floresco 2016; Bailey et al. 2016, 2020; Hart et al. 2017; Stutz et al. 2019). With these tasks, animals are offered the choice between lever pressing for a highly valued reinforcer vs. approaching and consuming a freely available but less preferred reinforcer. Tasks such as the concurrent fixed ratio 5 (FR5)/chow feeding choice task and the concurrent progressive ratio (PROG)/feeding choice task are useful for measuring effort-related choice behavior (Salamone et al. 2002, 2007, 2016a; Salamone and Correa, 2012; Randall et al. 2012). Several studies have shown that selection of the high-effort alternative is reduced when animals are exposed to manipulations associated with depression and negative motivational symptoms, such as stress (Shafiei et al. 2012; Bryce and Floresco, 2016; Dieterich et al. 2020), proinflammatory cytokine administration (Nunes et al. 2014; Yohn et al. 2015; Rotolo et al. 2020, in prep), and dopamine (DA) depletion or antagonism (Nunes et al. 2013; Randall et al. 2012, 2014; Yohn et al. 2015a; Hosking et al. 2015; Pardo et al. 2015; Yohn et al. 2016a,b,c; Rotolo et al. 2019; Yang et al. 2020; Bailey et al. 2020). For example, rats treated with the vesicular monoamine transport (VMAT-2) inhibitor tetrabenazine (TBZ) demonstrate a low-effort bias across several tasks, shifting choice away from the high-effort alternative (Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2015a; Pardo et al. 2015). When administered to humans, TBZ induces depressive symptoms including fatigue (Chitnis and Karunapuzha, 2009; Frank, 2010; Chen et al. 2012). Importantly, these effort-related dysfunctions in animals are not reversed by co-administration of

inhibitors of SERT or norepinephrine transport (NET) (Yohn et al. 2016a,b). Unlike drugs that inhibit SERT or NET, several inhibitors of the DA transporter (DAT), including bupropion, vanoxerine (GBR12909), lisdexamfetamine, PRX-14040, methylphenidate, and modafinil, can reverse the low-effort bias induced by TBZ (Nunes et al. 2013, Yohn et al. 2016a,b,c; Salamone et al. 2016b) and by pro-inflammatory cytokines (Yohn et al. 2016b,d). Moreover, several DAT inhibitors have demonstrated the ability to increase motivation to exert high levels of effort, increasing selection of lever pressing in rats tested on the PROG/chow feeding choice task when administered alone (Sommer et al. 2014; Randall et al., 2015; Yohn et al., 2016b,c,d).

The ability of drugs that block DAT to reverse effort-related impairments and increase selection of high-effort activities in rodents is consistent with clinical studies showing that DAT inhibitors, including bupropion, amphetamine, and methylphenidate, can improve motivational function in people (Stotz et al. 1999; Papakostas et al. 2006; Pae et al. 2007; Blockmans and Persoons 2016). The wakefulness agent modafinil, which inhibits DAT (Schmitt and Reith 2011), and elevates extracellular DA as measured by microdialysis (Mereu et al. 2017) and human imaging studies (Volkow et al. 2009), has been shown to improve motivational function in people with depression (Fava et al. 2007; Lam et al. 2007) and multiple sclerosis (Shangyan et al. 2018). Moreover, modafinil reduces perception of physical task demands during exercise (Rattray et al. 2019), and increases motivation and enhances task engagement in humans at doses that do not produce a powerful euphoria or ‘high’ (Müller et al. 2013). Modafinil has atypical DAT binding characteristics and behavioral effects (Schmitt and Reith 2011; Mereu et al. 2013, 2017; Cao et al. 2016; Nikiforuk et al. 2017), and is part of an emerging class of drugs (e.g. benztropine and GBR12909 analogs) that have binding properties and behavioral effects that are different from cocaine. Recently, various stereoisomers of the novel thiazole-based modafinil

analog CE-123 were demonstrated to have pro-cognitive effects in both rats and mice (Nikiforuk et al. 2017; Kristofova et al. 2018; Camats-Perna et al. 2019), as well as the ability to reverse the effort-related effects of TBZ in rats at doses that increase extracellular DA in nucleus accumbens core (Rotolo et al. 2019). While CE-123 has shown preclinical effectiveness across a variety of rodent behavioral tasks without adverse toxic effects (Kalaba et al. 2017), and has a higher specificity for the DAT than other commercially available drugs (Kalaba et al. 2020), it is important to provide a detailed characterization of a broad range of modafinil analogs to identify the most promising candidate for treating motivational dysfunctions and fatigue. The present study investigated a novel modafinil analog, (*S*, *S*)-CE-158, for its ability to bind to DAT, the specificity of its inhibition of DAT, and its ability to reverse the effort-related effects of TBZ, enhance high-effort PROG responding, and increase accumbens DA transmission.

## 4.2 Methods

*Animals*

Adult male, drug-naïve, Sprague Dawley rats (Envigo, Indianapolis, IN, USA) were housed in a colony maintained at 23 °C with 12-h light/dark cycles (lights on 07:00). Rats (n=23 for the behavioral pharmacology experiments; n=9 for the microdialysis experiment) weighed 275–299 g at the beginning of the study, and were initially food restricted to 85% of their free-feeding body weight for operant training. Rats were fed supplemental chow to maintain weight throughout the study, with water available ad libitum. Rats were allowed modest weight gain throughout the experiment. Animal protocols were approved by the University of Connecticut Animal Care and Use Committee, and followed NIH guidelines.

*In Vitro Binding Experiment*

To determine binding specificity, *in vitro* binding assays were conducted using transfected HEK293 cells with stable expression of human DAT with [N-Methyl-$^{3}$H]-WIN35,428 = [$^{3}$H]CFT = 2ß-carbomethoxy-3ß-(4-fluorophenyl) tropane. Cells were seeded in poly-D-lysine-coated 24-well plates (3 x $10^{4}$ HEK293 cells/well) and, 2 days later, binding on whole cells was performed as described previously (Pifl et al., 2009). Cells were incubated on ice for 2 h in 0.25ml buffer (mmol/l: 4 Tris–HCl; 6.25 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES); 120 NaCl; 5 KCl; 1.2 $CaCl_2$; 1.2 $MgSO_4$; 5 D-glucose; 0.5 ascorbic acid; pH 7.1) containing 6 nM [$^{3}$H]CFT and various concentrations of (*S, S*)-CE-158. Non-specific binding was measured in the presence of 10 µM mazindol. Binding experiments were performed by the laboratory of Dr. Gert Lubec.

*In Vitro Monoamine Transporter Reuptake Inhibition*

Monoamine transporter reuptake inhibition was outsourced to Eurofins DiscoverX Corporation (Fremont, CA) and performed according to the standard company protocol for the Neurotransmitter Transporter Uptake Assay (Molecular Devices). In short, (*S, S*)-CE-158 was tested for monoamine reuptake inhibition using a fluorescence-based assay. Fluorescent substrates mimicking amine neurotransmitters were taken up into HEK293 cells with stable expression of DAT, NET, and SERT, and intensity of intracellular fluorescence was quantified.

*Pharmacological Agents and Selection of Doses*

(*S, S*)-CE-158 (5-(((S)-((S)-(3-bromophenyl)(phenyl)methyl)sulfinyl)methyl)thiazole) (**Fig. 4.1**) was obtained from the Lubec Laboratory (University of Vienna, Austria) and dissolved in dimethyl sulfoxide (DMSO), Tween 80, and 0.9% saline. The DMSO/Tween 80/saline solution was administered as the vehicle control. TBZ (9,10-dimethoxy-3-(2-methylpropyl)-1,3,4,6,7, 11b hexahydrobenzo[a]quinolizin-2-one) was obtained from Tocris Bioscience (Ellisville, MO) and was dissolved in DMSO, 0.9% saline, and titrated with HCl. The DMSO/saline solution was administered as the vehicle control. The dose of 1.0 mg/kg TBZ was based on extensive piloting in our laboratory. The doses of (*S, S*)-CE-158 were selected based on extensive pilot studies and information about its relative affinity for DAT.

*Behavioral Procedures*

*Concurrent FR5/Chow Feeding Choice Task:*

Behavioral sessions were conducted in operant chambers (28 x 23 x 23 cm; Med Associates, Fairfax, VT). Sessions lasted 30 minutes a day for 5 days/week. First, rats were trained to lever press on a continuous reinforcement schedule to receive 45 mg high-carbohydrate pellets (Bio-Serv; Frenchtown, NJ, USA) for one week, then were shifted to the FR5 schedule. After 5 weeks of FR5 training, chow was introduced. During each FR5/chow feeding choice task session, 15-20 g of lab chow (Laboratory Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO) was concurrently available on the floor of the chamber. Rats were trained on this FR5/chow feeding choice procedure for 5 weeks, after which drug testing began. On baseline and drug treatment days, rats consumed all of the operant pellets that were delivered during each session.

*Concurrent PROG/Chow Feeding Choice Task:*

A second behavioral experiment was conducted to determine if (*S*, *S*)-CE-158 had an effect on rats' behavior on the PROG/chow feeding choice task when administered alone. Sessions were conducted in operant chambers (28 x 23 x 23 cm; Med Associates, Fairfax, VT) with 30-minute sessions 5 days/week. Rats were initially trained to lever press on a continuous reinforcement FR1 schedule (high-carbohydrate 45 mg pellets, Bio-Serv) and then shifted to the PROG schedule (Randall et al. 2012, 2014, 2015). For PROG sessions, the ratio started at FR1 and was increased by 1 additional response every time 15 reinforcements were obtained (FR1×15, FR2×15, etc.). A "time-out" feature deactivated the response lever for the rest of the session whenever 2 minutes elapsed without a completed ratio. After 9 weeks of training on the PROG schedule, chow was introduced and was concurrently available on the floor of the chamber during the PROG/chow feeding choice task sessions as previously described. Rats were trained on the PROG/chow feeding choice procedure for 5 weeks, after which drug testing began. On baseline and drug treatment days, rats consumed all of the operant pellets that were delivered during each session.

*Surgical Implantation of Dialysis Guide Cannulae*

Animals were anesthetized with an intraperitoneal injection of 100.0 mg/kg ketamine hydrochloride and 10.0 mg/kg xylazine prior to placement in a stereotaxic device (incisor bar 5.0 mm above interaural line). Microdialysis guide cannulae (Bioanalytical Systems) were implanted 2.0 mm dorsal to the accumbens core (AP +2.8 mm, ML +1.8 mm, DV −6.8 mm from bregma). Cannulae were surgically implanted in nine drug-naïve adult male Sprague Dawley rats, and

placements were counterbalanced, with four rats implanted on the left and five rats implanted on the right. After placement, guide cannulae were secured to the skull by stainless steel screws and cranioplastic cement. A stainless-steel stylet was inserted through each cannula while not in use. Rats were allowed 7 days to recover from surgery before undergoing dialysis procedures.

*DA Microdialysis and High Performance Liquid Chromatography*

On sample collection days, dialysis probes (Bioanalytical Systems; 2.0 mm active surface) were inserted through the microdialysis guide cannulae. Artificial CSF (aCSF; 147.2 mm NaCl, 2.4 mm $CaCl_2$, 4.0 mm KCl) was continuously perfused through the probe at a rate of 2.0 µl/min. Neurochemical samples were collected every 30 min in tubes containing 2.0 µl of ascorbic acid and sodium metabisulfite to prevent oxidation of DA. Up to 7 baseline samples were collected before an intraperitoneal injection of 8.0 mg/kg (*S, S*)-CE-158 to establish a stable DA level. The last three of those baseline samples were used as the statistical baseline. Samples were either immediately frozen or analyzed fresh using reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ESA Coulochem II system). The electrochemical parameters were as follows: channel 1 = −100 mV, channel 2 = +200 mV, and guard cell = +350 mV. Each liter of mobile phase contained 27.5 g sodium phosphate monobasic, 7.0 % methanol, 750 µl of 0.1 m EDTA, and 2200 µl of 0.4 m sodium octyl sulfate dissolved in deionized ultrapure $H_2O$ with a final pH of 4.5. The flow rate was 1.0 ml/min. DA standards were run each day before the dialysate samples. Probe placements were verified with histological analyses and only probes with placement in the nucleus accumbens core were used for analyses.

*Nissl Staining for Identifying Probe Placements*

At the completion of behavioral testing in the intracranial injection and microdialysis experiments, each animal was anesthetized with $CO_2$ and then perfused intracardially with physiological saline followed by a 3.7 % formaldehyde solution. The brains were removed and stored in formaldehyde and then sliced with a vibratome in 60 μm sections, which were mounted on glass microscope slides. After mounting, slides were stained with cresyl violet for microscopic observation by a blind observer. Any animal with improper cannulae placement or significant damage around the injection site was excluded from the statistical analyses of behavioral data.

*Experimental Procedures*

*Effects of systemic administration of TBZ on the concurrent FR5/chow feeding choice procedure and reversal with (S, S)-CE-158:*

Trained rats (n=7) were administered either TBZ (1.0 mg/kg) or vehicle, and (*S*, *S*)-CE-158 (2.0, 4.0, 8.0 mg/kg) or vehicle, via intraperitoneal (IP) injections on drug testing days. Rats received TBZ or vehicle 120 minutes before testing and (*S*, *S*)-CE-158 or vehicle 30 minutes before testing. The experiment used a within-groups design, with each rat receiving each drug treatment in a randomly varied order. The following five treatment combinations were given: TBZ vehicle + (*S*, *S*)-CE-158 vehicle; 1.0 mg/kg TBZ + (*S*, *S*)-CE-158 vehicle; 1.0 mg/kg TBZ + 2.0 mg/kg (*S*, *S*)-CE-158; 1.0 mg/kg TBZ + 4.0 mg/kg (*S*, *S*)-CE-158; 1.0 mg/kg TBZ + 8.0 mg/kg (*S*, *S*)-CE-158.

*Effects of systemic administration of (S, S)-CE-158 on the concurrent PROG/chow feeding choice procedure:*

Thirty minutes prior to the testing session, trained rats (n=16) were administered either vehicle or 2.0, 4.0, or 8.0 mg/kg (*S*, *S*)-CE-158. This experiment used a within-groups design, with each rat receiving each drug treatment in a randomly varied order, once per week over the course of four weeks.

*Effects of (S, S)-CE-158 on extracellular DA in the nucleus accumbens:*

Rats were implanted with dialysis probes in nucleus accumbens core as described earlier. On the test day, after baseline dialysis samples were collected, rats received intraperitoneal injections of either vehicle (n=4) or 8.0 mg/kg (*S*, *S*)-CE-158 (n=5). Seven post-injection samples were collected.

*Statistical Analysis*

Repeated measures ANOVA was used to determine the effect of drug treatment on lever pressing and chow intake in the behavioral pharmacology experiments. Since there were significant overall F values for the two behavioral measures being used, nonorthogonal planned comparisons were performed, using the overall error term to assess differences between each treatment and the control condition. The number of comparisons was restricted to the number of treatments minus one (Keppel, 1991). Statistical outliers were predefined as any point that is more than two standard deviations from the mean. No data from this study were excluded as outliers.

Changes in extracellular DA levels in the microdialysis experiment were calculated as the percent change from baseline, with the mean of the three samples immediately preceding the drug injections serving as the 100% baseline level. A 2 × 7 factorial ANOVA with the treatment (drug vs. vehicle) factor being between groups, and the sample factor (samples collected after drug injection) being repeated measures, was used to test for post-injection differences in extracellular levels of DA. The raw DA levels of the baseline samples were analyzed using t test to verify that the baseline DA levels were not different between conditions. Nonorthogonal planned comparisons were performed using the error term from the between-subjects analysis to assess differences between the two treatments at each particular sample.

## 4.3 Results

*Binding experiments on DAT expressing cells and inhibition of monoamine transporters (DAT, NET, SERT) by (S, S)-CE-158:*

(*S, S*)-CE-158 concentration-dependently displaced [$^3$H]CFT from human DAT with an $IC_{50}$ of 0.052 ± 0.017 µM (**Fig. 4.2**). As shown in **Figure 4.3**, (*S, S*)-CE-158 selectively and potently blocked DAT ($IC_{50}$ = 0.2271 µM), while having a weak effect on NET ($IC_{50}$ = 11.97 µM) and no effect on SERT.

*Ability of (S, S)-CE-158 to reverse the effects of TBZ on the concurrent FR5/chow feeding choice procedure:*

As shown in **Figure 4.4**, a repeated measures ANOVA revealed that there was an overall significant effect of drug treatment on lever pressing [$F(4,24)=34.940$, $p<0.001$]. Planned comparisons showed that TBZ significantly decreased lever pressing compared to vehicle treatment [$F(1,24)=63.675$, $p<0.001$], and that co-administration of the dose of 8.0 mg/kg (*S, S*)-CE-158 with TBZ significantly attenuated the effects of TBZ on lever pressing [$F(1,24)=39.06$, $p<0.001$]. A two-tailed t-test revealed no difference in lever pressing between the vehicle plus vehicle and TBZ plus (*S, S*)-CE-158 8.0 mg/kg treatments ($p=0.17$) (**Fig. 4.4a**). There was also a significant overall effect of drug treatment on chow intake [$F(4,24)=22.086$, $p<0.001$]. Additional planned comparisons revealed that TBZ alone significantly increased chow intake relative to vehicle treatment [$F(1,24)=38.098$, $p<0.001$], and that co-administration of 8.0 mg/kg (*S, S*)-CE-158 with TBZ significantly reduced chow intake compared to the TBZ plus vehicle condition [$F(1,24)=21.786$, $p<0.001$]. Furthermore, a two-tailed t-test revealed no difference in chow intake between the vehicle plus vehicle and TBZ plus 8.0 mg/kg (*S, S*)-CE-158 treatment groups ($p=0.42$) (**Fig. 4.4b**).

*Ability of (S, S)-CE-158 to increase high-effort responding on the concurrent PROG/chow feeding choice procedure:*

Repeated measures ANOVA revealed an overall main effect of drug treatment on lever pressing [$F(3,45)=3.583$, $p<0.05$], and planned comparisons demonstrated that lever presses were significantly increased at 4.0 mg/kg (*S, S*)-CE-158 [$F(1,45)=9.433$, $p<0.01$] and at 8.0 mg/kg (*S,*

*S*)-CE-158 [F(1,45)=5.54, p<0.05] compared to vehicle treatment (**Fig 4.5a**). Drug treatment with (*S, S*)-CE-158 also had a significant effect on chow intake during the PROG/chow session [F(3,45)=3.686, p<0.05], with a significant reduction in chow intake at the 4.0 mg/kg dose [F(1,45)=7.118, p<0.05] and at the 8.0 mg/kg dose [F(1,45)=5.613, p<0.05] compared to vehicle (**Fig. 4.5b**).

*Systemic administration of (S, S)-CE-158 increased extracellular DA in the nucleus accumbens:*

Administration of 8.0 mg/kg (*S, S*)-CE-158 significantly increased extracellular DA content in the nucleus accumbens (**Fig. 4.6**). Factorial ANOVA with repeated measures on the sample factor demonstrated that there was a significant overall difference across samples [F(6,42)=3.339, p<0.01] and a significant sample x treatment interaction [F(6,42)=3.011, p<0.05]. The factorial ANOVA also revealed a significant quadratic trend for the sample x treatment interaction [F(1,7)=912.422, p<0.01], which statistically characterized the tendency for DA levels to show a transient increase, followed by a decrease, after injection of (*S, S*)-CE-158. In addition, there was a significant overall difference between treatment groups across the seven samples [F(1,7)=703.162, p<0.001], showing that (*S, S*)-CE-158 produced a significant overall difference in DA across samples relative to vehicle injection. Nonorthogonal planned comparisons assessing the difference between the two treatments at each sample revealed significant differences at sample 2 (S2) [F(1,7)=14.376, p<0.01] and sample 3 (S3) [F(1,7)=10.509, p<0.05].

**4.4 Figures**

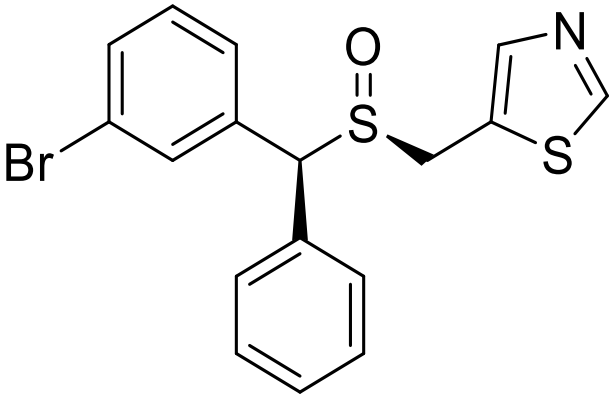



**Figure 4.1.** Chemical structure of (S, S)-CE-158 (5-(((S)-((S)-(3-bromophenyl)(phenyl)methyl)sulfinyl)methyl)thiazole).

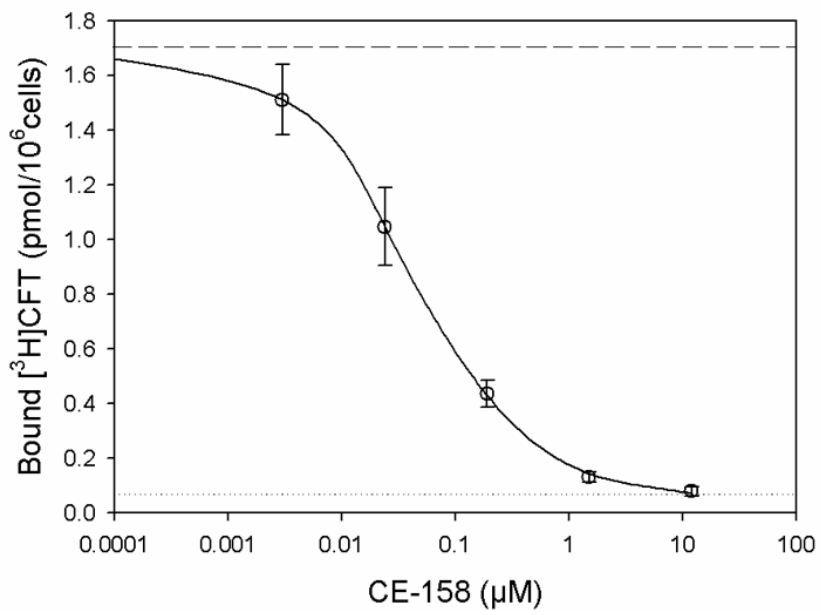



**Figure 4.2.** Effect of (S, S)-CE-158 on binding to DAT expressing cells. HEK293 cells with stable expression of the human DAT and seeded in 24-well plates were incubated with [3H]CFT for 2 hrs in the absence (dashed line) or presence of (S, S)-CE-158 at the concentration indicated (open circles, solid line) or 10 μM mazindol (dotted line) at 4°C, and binding of tritium was

determined as described under “Experimental procedures”. Symbols represent means of binding ± standard error of four independent experiments, each performed in duplicates.

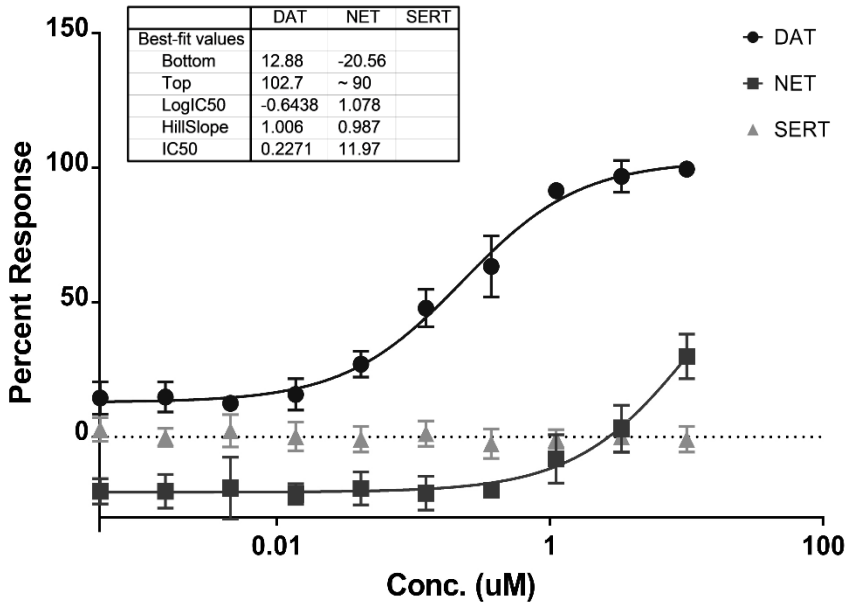



**Figure 4.3.** *In vitro* monoamine transporter reuptake inhibition of *(S, S)*-CE-158 using HEK293 cells with stable expression of DAT, NET, and SERT. *(S, S)*-CE-158 selectively and potently blocked DAT ($IC_{50}$ = 0.2271 µM), while having a weak effect on NET ($IC_{50}$ = 11.97 µM) and no effect on SERT. Symbols represent means of uptake inhibition ± standard error of three independent experiments, each performed in duplicates.

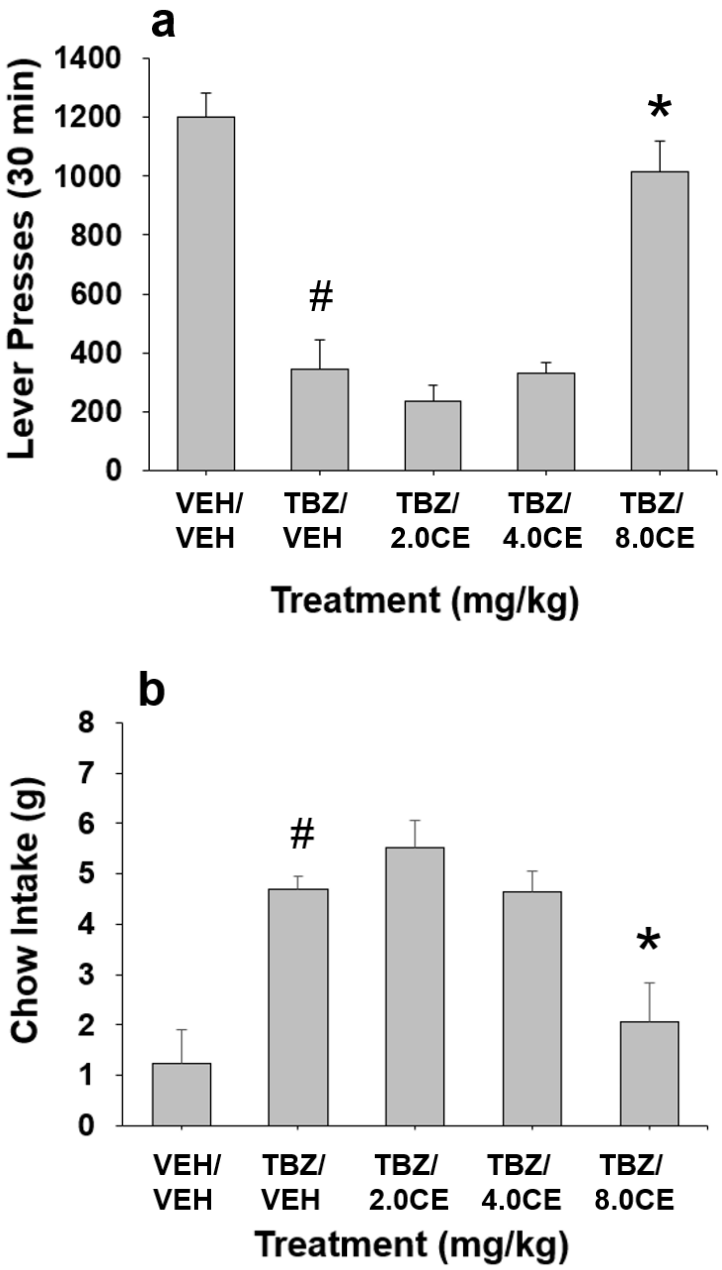



**Figure 4.4.** The effects of the DAT blocker (*S*, *S*)-CE-158 on TBZ-induced changes in performance on the concurrent lever pressing/chow-feeding choice procedure. **(a)** There was an overall significant effect of drug treatment on lever pressing ($p<0.001$). TBZ significantly decreased lever pressing compared to vehicle treatment (#$p<0.001$). **(b)** There was a significant overall effect of drug treatment on chow intake ($p<0.001$), and TBZ alone significantly increased

chow intake relative to vehicle treatment (#p<0.001). Co-administration of the dose of 8.0 mg/kg (*S*, *S*)-CE-158 with TBZ significantly attenuated the effects of TBZ on lever pressing (*p<0.001) and chow intake (*p<0.001).

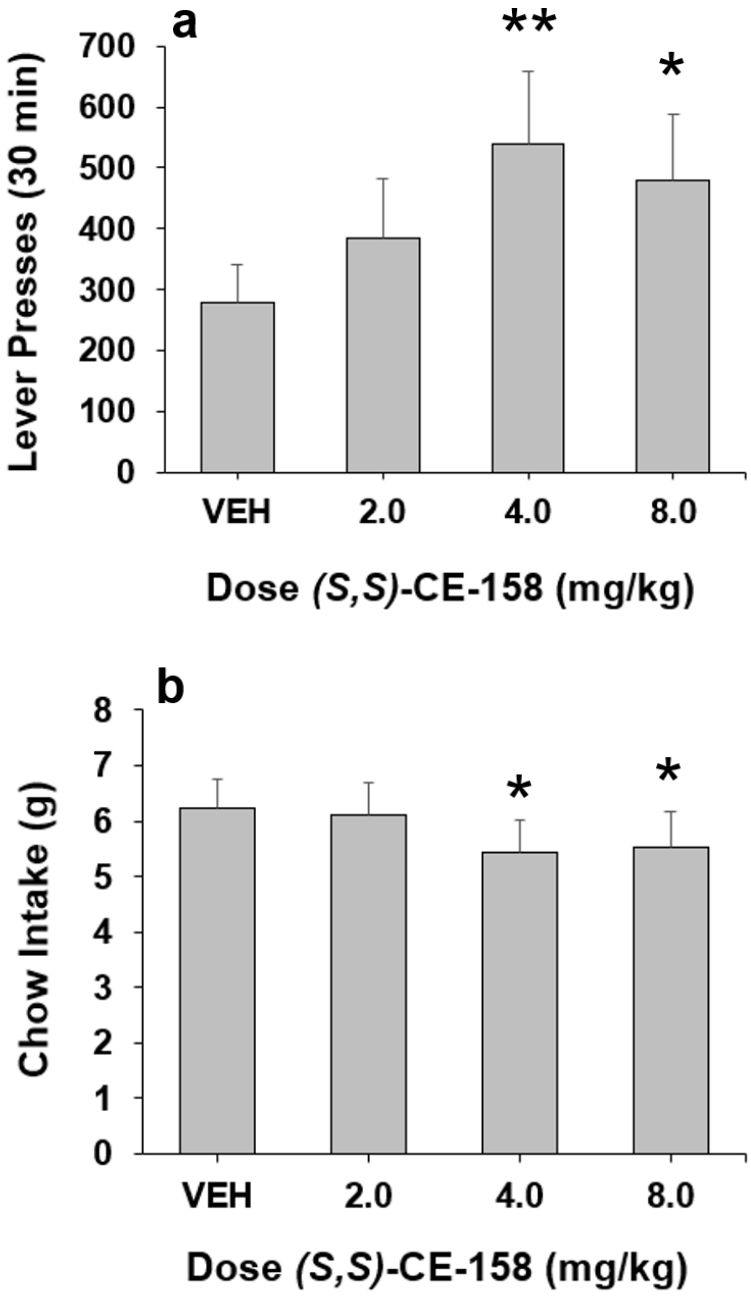


**Figure 4.5.** The effects of the DAT blocker (*S, S*)-CE-158 on performance on the concurrent lever pressing/chow-feeding choice procedure. **(a)** There was an overall main effect of drug treatment on lever presses ($p<0.05$). Lever presses were significantly increased at 4.0 mg/kg (*S, S*)-CE-158 (**$p<0.01$) and at 8.0 mg/kg (*S, S*)-CE-158 (*$p<0.05$) compared to vehicle treatment. **(b)** Drug treatment with (*S, S*)-CE-158 had a significant effect on chow intake during the PROG/chow session ($p<0.05$), with a significant reduction in chow intake at the 4.0 mg/kg dose (*$p<0.05$) and at the 8.0 mg/kg dose (*$p<0.05$) compared to vehicle.

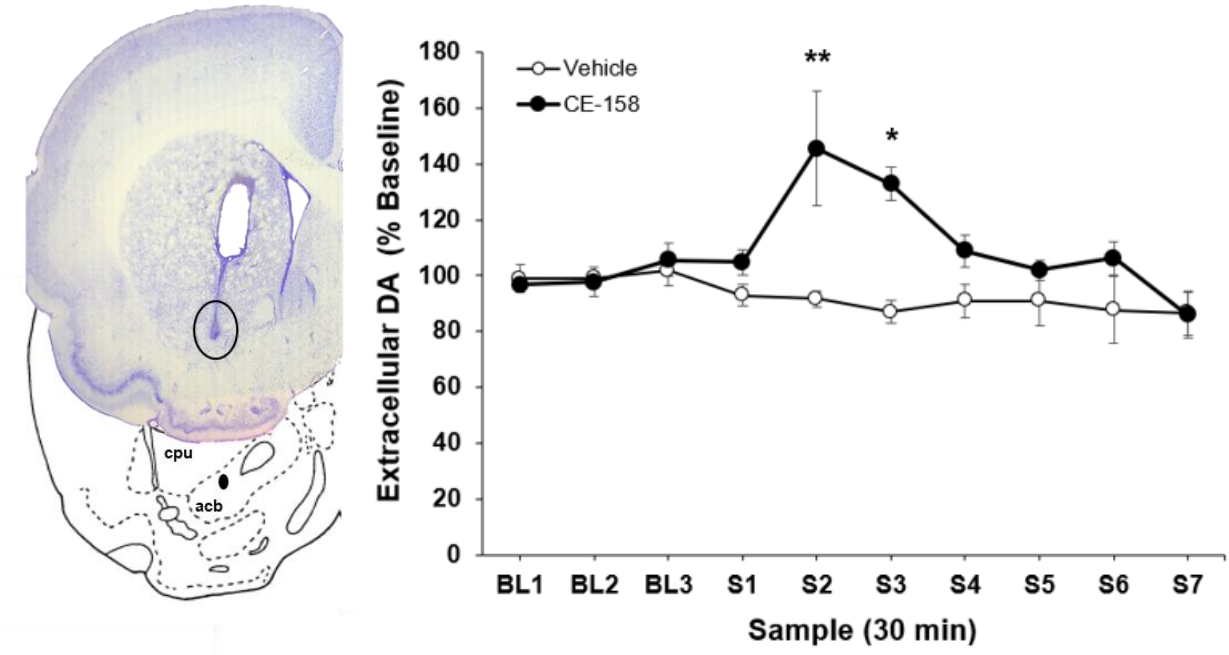



**Figure 4.6.** Effect of (*S, S*)-CE-158 on extracellular DA in the nucleus accumbens. Left: Photomicrograph and schematic showing a representative probe placement in nucleus accumbens. Abbreviations: acb: nucleus accumbens; cpu: caudate nucleus-putamen (neostriatum) (Pellegrino et al. 1979). Right: Mean (± SEM) extracellular DA (expressed as percent baseline) in 30-min samples. Three baseline (BL) samples were collected prior to

injection of vehicle or 8 mg/kg (*S, S*)-CE-158, followed by seven post-drug samples (S1-7). **p<0.001, (*S, S*)-CE-158 significantly differs from vehicle at S2. *p<0.05, (S, S)-CE-158 significantly differs from vehicle at S3. #p<0.05 significant overall difference between vehicle and (*S, S*)-CE-158 across the seven post-drug samples.

## Chapter 5: The Novel Atypical Dopamine Transport Inhibitor CT-005404 Reverses the Effort-Related Motivational Effects of Tetrabenazine and Interleukin-1β and Increases Progressive Ratio Responding

Submitted; Rotolo et al., *Neuropharmacology*

### 5.1 Introduction

Patients with major depressive disorder (MDD), Parkinsonism, schizophrenia, multiple sclerosis (MS) and other psychiatric or neurological disorders commonly experience motivational symptoms such as fatigue and loss of energy (Feinstein, 2007; Fava et al., 2014). According to the DSM-V, one of the nine core symptoms of MDD is "fatigue or loss of energy nearly every day", and this is the second most frequently reported symptom (Zimmerman et al. 2015; Tolentino and Schmidt, 2018). Fatigue and anergia are demonstrated by an individual's lack of energy or willingness to work for something that is significant (Treadway et al. 2012; Yang et al. 2014; Zimmerman et al. 2015; Corfield et al. 2016), which has been demonstrated by the tendency of individuals with depression, Parkinsonism, and schizophrenia to show a low-effort bias when tested on effort-related choice procedures (Treadway et al. 2012; Yang et al. 2014; Chong et al. 2016; Barch et al. 2017). The risk of depression in individuals who report fatigue is two-fold greater compared to non-fatigued individuals (Corfield et al. 2016). Historically, serotonin transport (SERT) inhibitors have been the most commonly prescribed drugs for treating MDD patients, but SERT blockers are relatively ineffective at improving anergia or fatigue-related aspects of motivation (Cooper et al., 2014; Fava et al. 2014; Rothschild et al. 2014). In contrast, evidence indicates that the catecholamine uptake inhibitor bupropion

(Pae et al., 2007; Papakostas et al., 2006; Cooper et al., 2014), and drugs that inhibit the dopamine (DA) transporter (DAT) such as d-amphetamine and methylphenidate (Stotz et al., 1999; Hanna et al. 2006), can partially alleviate these symptoms in depressed patients. Bupropion also was reported to reduce fatigue associated with MS (Duffy and Campbell 1994).

Given the clinical importance of motivational dysfunctions in psychopathology, it is vital to employ animal models that are useful for drug development. Thus, several recent studies have focused on rodent tasks involving effort-related choice behavior. For assessment of effort-based choice, animals are offered the option of a high-effort instrumental action leading to highly valued reinforcer (preferred, or higher magnitude) vs. a low effort/low reward choice. A low-effort bias in rodents can be induced by several conditions associated with motivational dysfunction in depression and other disorders, including stress (Shafiei et al. 2012; Bryce and Floresco 2016) and inflammatory challenge (Nunes et al. 2014; Yohn et al. 2016b,d). DA antagonists or depletions reliably produce a low-effort bias in rodents (Salamone et al. 1991, 2002; Mai et al. 2012; Pardo et al. 2012; Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2015a,b; Yang et al. 2020). Tetrabenazine (TBZ) is an inhibitor of the vesicular monoamine transporter type-2 (VMAT-2), which blocks vesicular storage and depletes monoamines, though its greatest effects at low doses are on striatal and accumbens DA (Pettibone et al. 1984; Tanra et al. 1995; Nunes et al. 2013). TBZ has been shown to produce depressive symptoms including anergia and fatigue in humans (Frank 2009, 2010; Guay 2010; Chen et al. 2012), and thus it has been used in numerous preclinical experiments involving effort-based choice to induce a low-effort bias (Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2015, 2016a,b,c; Salamone et al. 2016b; Carratala-Ros et al. 2020). TBZ-induced shifts in effort-based choice are not due to changes in food intake or preference, sucrose preference or hedonic reactivity, or changes in

reference memory, and the effects of TBZ do not resemble those produced by appetite suppressant drugs or reinforcer devaluation (Nunes et al. 2013; Randall et al. 2014; Pardo et al. 2015; Yohn et al. 2015, 2016a,b,c; Salamone et al. 2016b; Yang et al. 2020; Carratala-Ros et al. 2020). The low-effort bias induced by TBZ in rats was not reversed by the commonly prescribed SERT inhibitors fluoxetine or citalopram (Yohn et al. 2016a,b), but it was attenuated by several drugs that block DAT, including bupropion, GBR12909, PRX-14040, lisdexamfetamine, *(S)*-CE-123, methylphenidate, and modafinil (Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2015, 2016a,b,c; Salamone et al. 2016a,b; Rotolo et al. 2019).

Although there are classical DAT inhibitors such as cocaine, and drugs that block DAT and release DA such as amphetamines and methylphenidate, there is an emerging interest in the neurochemical and behavioral characteristics of novel atypical DAT blockers, such as *N*-substituted benztropine analogs and modafinil analogs (Schmitt and Reith, 2011; Zhang et al. 2017; Rotolo et al. 2019). In contrast to cocaine and amphetamines, which rapidly increase extracellular DA in nucleus accumbens (Tanda et al. 1997; Desai et al. 2010), GBR 12909 and some benztropine analogs induce a relatively slow increase in extracellular DA over a broad time course (Raje et al. 2003; Desai et al. 2005a,b, 2010; Tanda et al, 2005, 2009, 2013; Kohut et al. 2014), which may offer advantages in terms of clinical utility. In view of the potential importance of DAT inhibition as a target for developing drugs to treat motivational dysfunction, the present paper provides an initial characterization of the neurochemical and effort-related behavioral effects of a novel atypical DAT inhibitor, CT-005404. As well as providing data on DAT binding and alterations in extracellular DA as measured by microdialysis, the present studies evaluated the ability of CT-005404 to reverse the effort-related impairment induced by TBZ and the suppression of lever pressing induced by the pro-inflammatory cytokine IL-1β in

rats tested on the concurrent fixed ratio 5 (FR5)/chow feeding choice task. Furthermore, CT-005404 was assessed for its ability to increase selection of high-effort lever pressing in rats tested on the progressive ratio (PROG)/chow feeding choice task.

## 5.2 Materials and Methods

*Animals*

Sprague Dawley rats were obtained from Charles River Laboratories (Germany) and divided into 6 groups (n=4/group; 220-250 g) for the *in vivo* receptor occupancy experiment. Fifteen adult males (328-485 g) were used for the microdialysis experiments. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2011), European Union directive 2010/63, and Dutch law. Experiments were licensed by the national committee for animal experiments (AVD247002015341; Centrale Commissie Dierproeven) and approved by the Animal Care and Use Committee of Charles River Discovery Groningen. Animals were housed in groups of 5 in polypropylene cages (40 X 50 X 20 cm) with wire mesh tops in a temperature (22 ± 2 °C) and humidity (55 ± 15%) controlled environment (12-h light/dark cycle, lights on 07:00). Standard diet (SDS Diets, RM1 PL) and water were available ad libitum.

For the behavioral pharmacology experiments, adult male Sprague Dawley rats (Envigo, Indianapolis, IN, USA; 279-299 g) were pair-housed in a colony maintained at 23°C (12-h light/dark cycle, lights on at 07:00). Rats were initially food restricted to 85% of their free-feeding body weight for operant training and then allowed modest growth, with water available ad libitum in the home cages for the duration of the experiment. Protocols were approved by the

University of Connecticut Institutional Animal Care and Use Committee, and studies were conducted according to NIH guidelines.

*CT-005404 In Vivo Receptor Occupancy*

Rats were treated with either vehicle (0.5% (w/v) (hydroxypropyl)methyl cellulose (HPMC) in 0.9% saline) or CT-005404 (1.0, 3.0, 10.0, 30.0, or 100.0 mg/kg), and 100 minutes later were given [$^{18}$F]FECNT (8-(2-[$^{18}$F]fluroethyl)-2betacarbomethoxy-3beta-(4-chlorophenyl)nortropane) (i.v. 3.0 µg/kg, 0.5 ml/kg). After the tracer survival interval, rats were euthanized by cervical dislocation. Trunk blood was collected in K2-EDTA coated tubes and stored on ice. Whole brains were removed and rinsed with sterile water. Target tissues (striatum and cerebellum), were dissected, weighed, and stored in 1.5 ml tubes on dry ice at -80°C until tissue extraction. Blood samples were centrifuged at 1650 x g for 16 minutes, and serum was pipetted into tubes and stored at -80°C. Receptor occupancy experiments were performed by Chronos Therapeutics laboratories.

*In Vivo Microdialysis*

*Surgical Implantation of Dialysis Guide Cannulae:*

Rats were anesthetized using isoflurane (2% and 500 mL/min O2). Before surgery, Finadyne analgesia (1.0 mg/kg, s.c.) was administered. A bupivacaine/epinephrine mixture was used for local analgesia of the incision site. Animals were placed in a stereotaxic apparatus (Kopf instruments, USA) with the incisor bar at -3.0 mm, and guides for I-shaped microdialysis probes with 2.0 mm exposed surface (polyacrylonitrile membrane; CRL, the Netherlands) were implanted into nucleus accumbens (coordinates: AP = +1.6 mm (from bregma), lateral +/-1.8

mm (from midline), ventral -7.7 mm (from dura), based on Paxinos and Watson (2008)). Cannulae were attached to the skull with stainless steel screws and dental cement.

*DA Microdialysis:*

Experiments were initiated one day after cannulae implantation. Microdialysis probes were connected with PEEK tubing (Western Analytical Products Inc. USA; PK005-020) to a microperfusion pump (Harvard Apparatus) and perfused with artificial CSF (aCSF: 147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$) at 1.5 µl/min flow rate. After two hours of stabilization, microdialysis samples were collected in 20-minute intervals. After the third basal sample (t=0 minutes) vehicle or 10.0 or 30.0 mg/kg CT-005404 (0.5% (w/v) (hydroxypropyl)methyl cellulose (HPMC) in 0.9% saline) were orally administered. Microdialysates were collected for 360 minutes after compound administration. Samples were collected into polystyrene microvials (Microbiotech/se AB, Sweden; 4001029) using an automated collector (UV 8301501, TSE, Univentor, Malta). The vials contained 10 µL stabilization fluid (0.02 M formic acid + 0.04% ascorbic acid in ultrapurified water). Samples were stored at -80 °C. After the experiment animals were sacrificed, and brain tissue was collected for verification of probe placements. Microdialysis experiments were performed by Chronos Therapeutics laboratories.

*Analytical Procedure:*

The volume for the microdialysis samples was 40 µL. Concentrations of DA were determined by HPLC with tandem mass spectrometry (MS/MS) detection using the following internal standards (IS): D4-DA for DA. An aliquot of IS solution was mixed with an aliquot of

each sample. The resulting mixture was derivatized with SymDAQ™ automatically in the autosampler and then injected onto the HPLC by an automated sample injector (Shimadzu, Japan). Chromatographic separation was performed with a Phenomenex, Synergi MAX-RP column (100 x 3 mm, 2.5 µm; 35 °C). The mobile phases consisted of A: ultrapurified water+0.2% acetonitrile+0.1% formic acid and B: 70% acetonitrile in ultrapurified water+0.1% formic acid. Elution of compounds proceeded using a linear gradient (flow rate- 0.3 mL/min). The MS analyses were performed using an API 4000 MS/MS system (API 4000 MS/MS detector, Turbo Ion Spray interface, Applied Biosystems, The Netherlands). The acquisitions were performed in positive ionization mode with the instrument operated in multiple-reaction-monitoring mode.

*Behavioral Pharmacology Procedures*

*FR5/Chow Feeding Choice Task:*

Behavioral sessions (30 minutes/day, 5 days/week) were conducted in operant chambers (28 x 23 x 23 cm; Med Associates, Fairfax, VT). First, rats were trained to lever press on a FR1 schedule reinforced by 45 mg high-carbohydrate pellets (Bio-Serv; Frenchtown, NJ, USA) for one week, then were shifted to the FR5 schedule. After 5 weeks of FR5 training, chow was introduced. During FR5/chow feeding choice sessions, 15-20 g of lab chow (Laboratory Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO) was concurrently available on the floor of the chamber. At the end of each session, rats were removed from operant chambers, the number of lever presses was recorded, and the remainder of chow and spillage in the chamber floor was weighed and recorded. Rats received FR5/chow feeding choice training for 5 weeks, after which

drug testing began. On baseline and drug treatment days, rats consumed all the operant pellets that were delivered during each session.

*PROG/Chow Feeding Choice Task:*

Behavioral sessions (30 minutes/day, 5 days/week) were conducted in operant chambers. Rats were initially trained to lever press on a FR1 schedule on Bio-Serv pellets as described above, then were shifted to the PROG schedule. For PROG sessions, the ratio started at FR1 and was increased by one additional response every time 15 reinforcers were obtained. A "timeout" feature deactivated the response lever and turned off the house light for the rest of the session whenever 2 min elapsed without a completed ratio. After 9 weeks of PROG training, chow was introduced. During each session, 15-20 g of lab chow (Laboratory Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO) was concurrently available on the floor of the chamber. At the end of each session, rats were removed from operant chambers, the number of lever presses was recorded, and the chow intake was determined. Rats were trained on this PROG/chow feeding choice procedure for 5 weeks, after which drug testing began. On baseline and drug treatment days, rats consumed all of the operant pellets that were delivered during each session.

*Behavioral Pharmacology Experiments*

*Drug Treatments and Dose Selection:*

CT-005404 was obtained from Chronos Therapeutics (Oxford, UK), and was dissolved in dimethylsulfoxide (DMSO) (1%) and hydroxypropyl methylcellulose (HPMC) (99%). The DMSO/HPMC solution was administered as the vehicle control. For Exp. 1, TBZ (9,10-dimethoxy- 3-(2-methylpropyl)-1,3,4,6,7, 11b hexahydrobenzo[a]quinolizin- 2-one) was

obtained from Tocris Bioscience (Ellisville, MO), and was dissolved in DMSO (20%) and 0.9% sterile saline (20%). Once partially dissolved, the TBZ solution was titrated with microliter quantities of 1.0 N HCl to fully dissolve the drug at a pH of 4.0-4.5. The dose of 1.0 mg/kg TBZ was based on previous studies (Rotolo et al. 2019). For Exp. 2, IL-1β was obtained from R&D Systems (Minneapolis, MN, USA), and was dissolved in 0.9% saline, which also served as the vehicle control. The dose of IL-1β was based on previously published data (Merali et al., 2003; Nunes et al. 2014) and pilot studies. The doses of CT-005404 were selected based on target engagement pilot studies (e.g. time course of receptor occupancy and receptor occupancy by dose) conducted by Chronos Therapeutics (see Figure 1).

*Behavioral Pharmacology Experiments:*

Rats (n=15) trained on the FR5/chow choice feeding procedure were administered either TBZ (1.0 mg/kg i.p.) or vehicle and CT-005404 (7.5, 15.0, and 30.0 mg/kg p.o.) or vehicle on drug testing days. CT-005404 or vehicle was administered 240 min (n=7) or 180 min (n=8) before testing, and TBZ or vehicle was administered 120 min before testing. A within-subjects design was used, with each animal receiving each drug treatment in a randomly varied order. Treatment combinations were as follows: TBZ vehicle + CT-005404 vehicle (control); 1.0 mg/kg TBZ + CT-005404 vehicle; 1.0 mg/kg TBZ + 7.5 mg/kg CT-005404; 1.0 mg/kg TBZ + 15.0 mg/kg CT-005404; 1.0 mg/kg TBZ + 30.0 mg/kg CT-005404. Drug treatments were given once per week, with none of the treatment sequences repeated across different animals.

For the CT-005404/IL-1β reversal study, a second group of trained rats (n=11) were administered either IL-1β (4.0 μg/kg i.p.) or vehicle and CT-005404 (7.5, 15.0, and 30.0 mg/kg p.o.) or vehicle on drug testing days. CT-005404 or vehicle was administered 240 min before

testing, and IL-1β or vehicle was administered 90 min before testing. A within-subjects design was used, with each animal receiving each drug treatment in a randomly varied order. Treatment combinations were: IL-1β vehicle + CT-005404 vehicle (control); 4.0 μg/kg IL-1β + CT-005404 vehicle; 4.0 μg/kg IL-1β + 7.5 mg/kg CT-005404; 4.0 μg/kg IL-1β + 15.0 mg/kg CT-005404; 4.0 μg/kg IL-1β + 30.0 mg/kg CT-005404. Drug treatments were given once per week, with none of the treatment sequences repeated across different animals.

For the CT-005404 PROG/chow feeding choice study, a group of rats (n=16) trained on the PROG/chow feeding choice procedure received 7.5, 15.0, or 30.0 mg/kg CT-005404 or vehicle 240 min before testing. Treatments were given once per week, in a randomly varied order, over the course of 4 weeks.

*Statistical Analysis*

For evaluation of the *in vivo* receptor occupancy data, a [agonist] vs. response variable slope model was used. The dose required to produce 50% occupancy (Occ50) and SEM was calculated using GraphPad Prism 8.4.1.

Changes in extracellular DA levels in the microdialysis experiment were calculated as the percent change from baseline, with the mean of the three samples immediately preceding the drug injections serving as the 100% baseline level. Data from one rat was excluded due to interrupted sample collection. Data from a total of 14 rats were included in the analysis (vehicle n=4, 10.0 mg/kg n=5, 30.0 mg/kg n=5). All post-administration samples were expressed as a percentage of basal level within the same subject. A 3 × 17 factorial ANOVA with the treatment (two doses of CT-005404 vs. vehicle) factor being between groups, and the sample factor (samples collected after drug injection) being repeated measures, was used to test for post-

injection differences in extracellular levels of DA. The raw DA levels of the baseline samples were analyzed using t tests to verify that the baseline DA levels were not different between conditions. Dunnett's tests using the error term from the between-subjects analysis were used to assess differences between each drug treatment and vehicle at each particular sample.

For the behavioral pharmacology experiments, total number of lever presses and gram quantity of chow intake were analyzed using repeated measures ANOVA. Nonorthogonal planned comparisons restricted to the number of treatments minus 1 (Keppel, 1991) using the overall error term were performed when the repeated measures ANOVA yielded a significant F value, to assess differences between each treatment and the control condition. The level of statistical significance was defined *a priori* at $p<0.05$. SPSS Statistics (Version 25), was used to perform all statistical analyses.

## 5.3 Results

*CT-005404 In Vivo Receptor Occupancy:*

*In vivo* receptor occupancy studies were performed. Occupancy of the DAT as it relates to dose was calculated for CT-005404. It was determined that the Occ50 of CT-005404 was 8.224 mg/kg (± SEM 1.646) (**Fig. 5.1A**). Receptor occupancy was measured after CT-005404 administration at 0.5, 1, 2, 4, 8, 12, and 24 hours. The peak receptor occupancy percentage was observed 4 hours post-treatment with a 78% occupancy (± 2.2 SEM) (**Fig. 5.1B**). **Table 5.1** shows the in vitro binding affinities for CT-005404 at DAT, NET, and SERT binding sites. CT-005404 showed an 80-fold selectivity for binding to the DAT relative to the NET, and a 1600-fold selectivity for DAT vs. SERT.

*In Vivo Microdialysis:*

Administration of CT-005404 significantly increased extracellular DA content in the nucleus accumbens (**Fig. 5.1C**). Factorial ANOVA with repeated measures on the sample factor demonstrated that there was a significant overall difference across samples [$F(16,176)=13.58$, $p<0.001$] and a significant sample x treatment interaction [$F(32,176)=2.90$, $p<0.001$]. In addition, administration of CT-005404 yielded a significant overall main effect of treatment group on extracellular DA across the seventeen post-injection samples [$F(2,11)=7.03$, $p<0.05$]. Simple effects of treatment group were assessed by computing between groups ANOVAs at each time point. For each significant between groups overall ANOVA, post-hoc two-tailed Dunnett's tests were performed, which revealed significant differences in extracellular DA for both the 10.0 mg/kg and 30.0 mg/kg treatment groups compared to vehicle from 80-280 min post-injection (with the exception of the 180-minute time point due to increased error).

*Behavioral Pharmacology Experiments*

*FR5/Chow Feeding Choice Task:*

**Figure 5.2** shows the effects of CT-005404 on TBZ-induced changes in rats tested on the FR5/chow feeding choice procedure. CT-005404 was administered either 3 hours or 4 hours prior to testing in two different groups of rats. In both groups, TBZ shifted effort-based choice, decreasing lever pressing and increasing chow intake. A treatment x lead time repeated measures factorial ANOVA revealed a significant overall main effect of treatment on lever pressing [$F(4,52)=32.28$, $p<0.001$], but no effect of lead time on lever pressing [$F(1,13)=0.39$, p=n.s.], and no significant treatment x lead time interaction [$F(4,52)=0.824$, p=n.s.] (**Fig. 5.2A**). Due to a non-significant interaction, non-orthogonal planned comparisons were computed collapsed

across the 3- and 4-hour lead times. TBZ+vehicle significantly reduced lever presses relative to the vehicle+vehicle treatment [$F(1,52)=102.682$, $p<0.001$]. Planned comparisons revealed a significant increase in lever presses for both the TBZ+15.0 mg/kg CT-005404 [$F(1,52)=9.844$, $p<0.01$] and TBZ+30.0 mg/kg CT-005404 [$F(1,56)=9.068$, $p<0.01$] relative to the TBZ+vehicle treatment (**Fig. 5.2A**). Factorial ANOVA assessing chow intake revealed a significant treatment effect [$F(4,52)=15.399$, $p<0.001$], but no effect of lead time on chow intake [$F(1,13)=2.268$, p=n.s.], and no significant treatment x lead time interaction [$F(4,52)=0.800$, p=n.s.] (**Fig. 5.2B**). TBZ+vehicle significantly increased chow intake relative to vehicle+vehicle treatment [$F(1,52)=47.645$, $p<0.001$]. Planned comparisons revealed a significant decrease in chow intake for both the TBZ+15.0 mg/kg CT-005404 [$F(1,52)=4.829$, $p<0.05$] and TBZ+30.0 mg/kg CT-005404 [$F(1,52)=18.495$, $p<0.001$] relative to the TBZ+vehicle treatment (**Fig. 5.2B**).

For the CT-005404/IL-1β reversal study, repeated measures ANOVA demonstrated a significant overall treatment effect on lever presses [$F(4,40)=12.504$, $p<0.001$] (**Fig. 5.3A**). IL-1β+vehicle significantly reduced lever presses compared to vehicle+vehicle [$F(1,40)=40.759$, $p<0.001$]. Planned comparisons revealed that treatment with both 15.0 mg/kg CT-005404 and 30.0 mg/kg CT-005404 were able to significantly increase lever presses from IL-1β plus vehicle treatment [$F(1,40)=4.180$, $p<0.05$; $F(1,40)=8.992$, $p<0.01$, respectively] (**Fig. 5.3A**). There was no overall treatment effect for chow intake [$F(4,40)=1.558$, p=n.s.] (**Fig. 5.3B**).

*PROG/Chow Feeding Choice Task*

Repeated measures ANOVA revealed for the effect of CT-005404 on lever pressing closely approached statistical significance [$F(3,45)=2.628$, $0.1 < p > 0.05$] (**Fig. 5.4A**), and a statistically significant effect of CT-005404 on chow intake was found [$F(3,45)=8.835$, $p<0.01$]

(**Fig. 5.4B**). However, orthogonal analysis of trend showed that there were significant linear dose-related effects for lever presses [$F(1,15)=6.632$, $p<0.05$] and chow intake [$F(1,15)=12.035$, $p<0.01$]. Because the linear dose-related trend was significant, planned comparisons were computed for number of lever presses. Rats treated with 30.0 mg/kg CT-005404 lever pressed significantly more [$F(1,45)=6.617$, $p<0.05$] and consumed less chow [$F(1,45)=11.240$, $p<0.01$] than rats treated with vehicle. Rats treated with 15.0 mg/kg CT-005404 also consumed significantly less chow during the session compared to vehicle [$F(1,45)=7.911$, $p<0.01$]. Moreover, a significant negative correlation was found when the percent change in lever presses was compared to the percent change in chow intake from vehicle at the 15.0 mg/kg treatment dose (Pearson's $r = -0.538$, $p<0.05$).

## 5.4 Figures

| Compound | DAT | NET | SERT |
|---|---|---|---|
| CT-005404 | 5nM | 398nM | 8μM |

**Table 5.1.** Binding affinity of CT-005404 to the DA transporter (DAT), the norepinephrine transporter (NET), and the 5-HT transporter (SERT). Values represent IC50s calculated from means of technical replicates in a 10 point dose response curve.

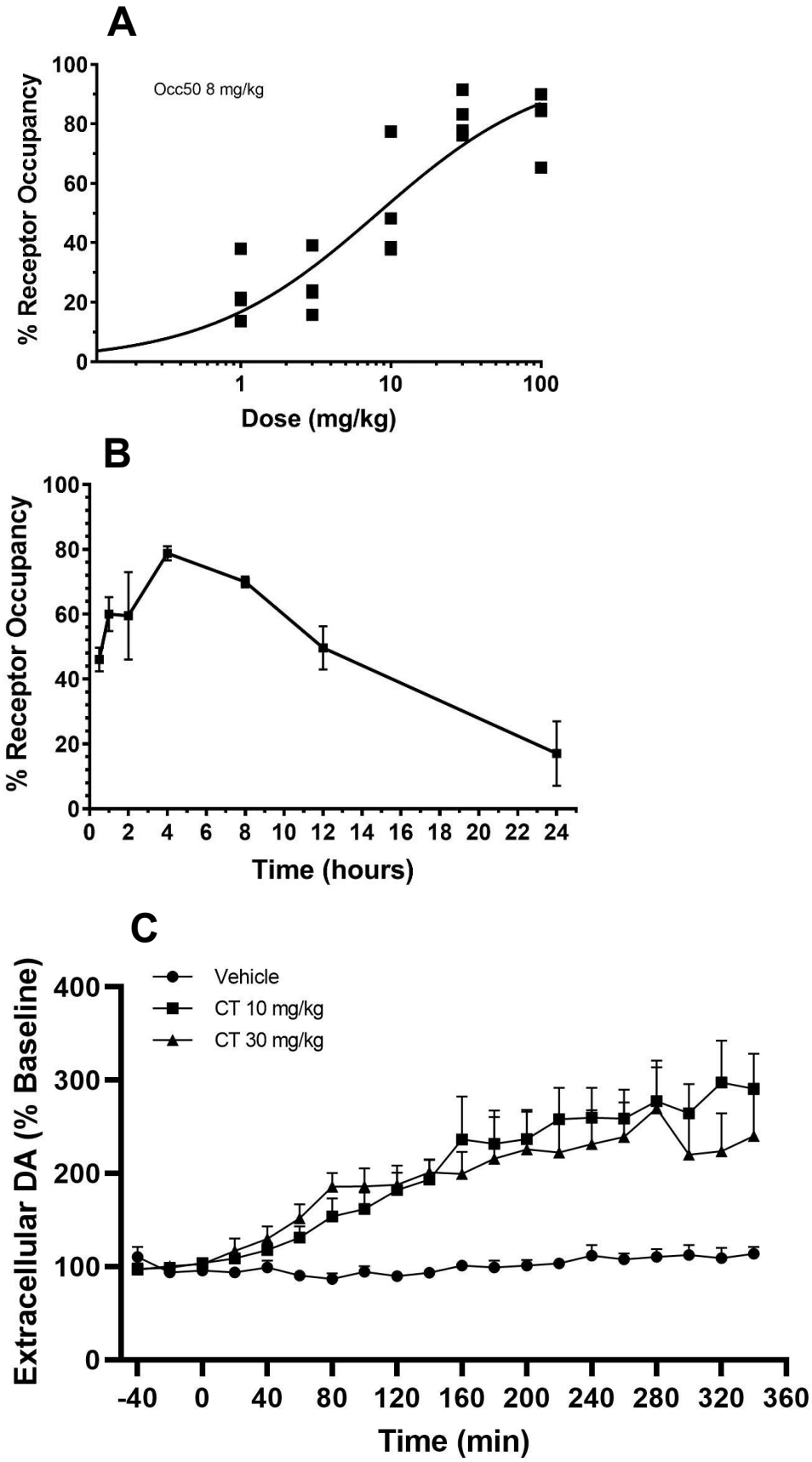
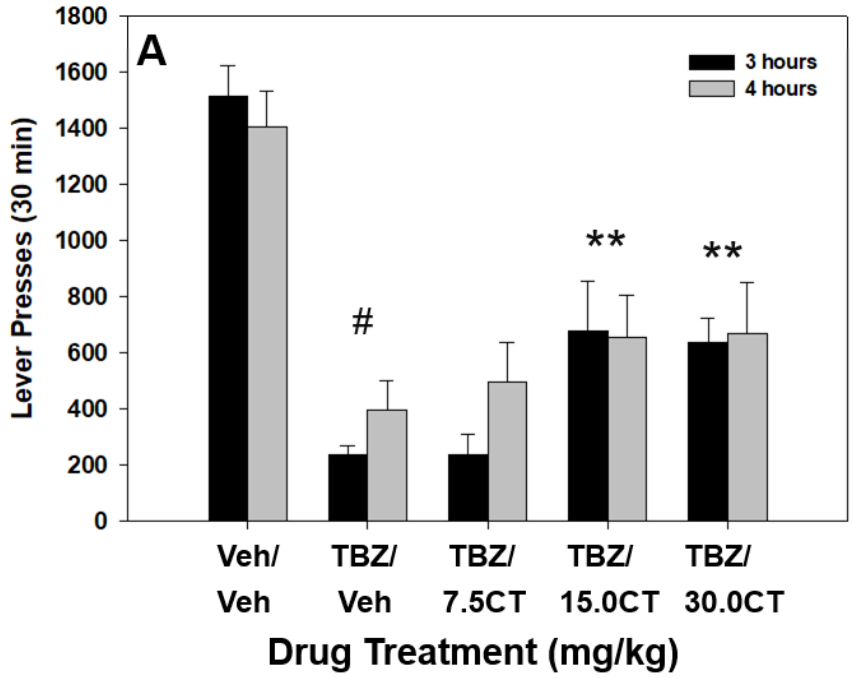

A
Occ50 8 mg/kg
% Receptor Occupancy
Dose (mg/kg)
B
% Receptor Occupancy
Time (hours)
C
Vehicle
CT 10 mg/kg
CT 30 mg/kg
Extracellular DA (% Baseline)
Time (min)

**Figure 5.1.** DAT occupancy and extracellular levels of DA in nucleus accumbens after oral administration of CT-005404. (A) DAT occupancy as it relates to dose (mg/kg). The dose of CT-005404 required to produce 50% occupancy (Occ50) of the DAT was 8.224 mg/kg (± 1.6 SEM). (B) DAT occupancy over time (hours). DAT occupancy was measured after CT-005404 administration at 0.5, 1, 2, 4, 8, 12, and 24 hours. The peak % DAT occupancy was observed 4 hours post-treatment with a 78% occupancy (± 2.2 SEM). (C) Microdialysis data showing extracellular DA in nucleus accumbens after p.o. administration of CT-005404. Mean (± SEM) extracellular DA (expressed as percent baseline) in 20-min samples. Three baseline samples were collected prior to injection of vehicle or 10.0 or 30.0 mg/kg CT-005404, followed by 17 post-injection samples. There was a significant overall effect of treatment group across the 17 post-drug samples, and a significant group x sample interaction. †$p<0.05$, 30.0 mg/kg CT-005404 significantly different from vehicle. *$p<0.05$, 10.0 mg/kg significantly different from vehicle.



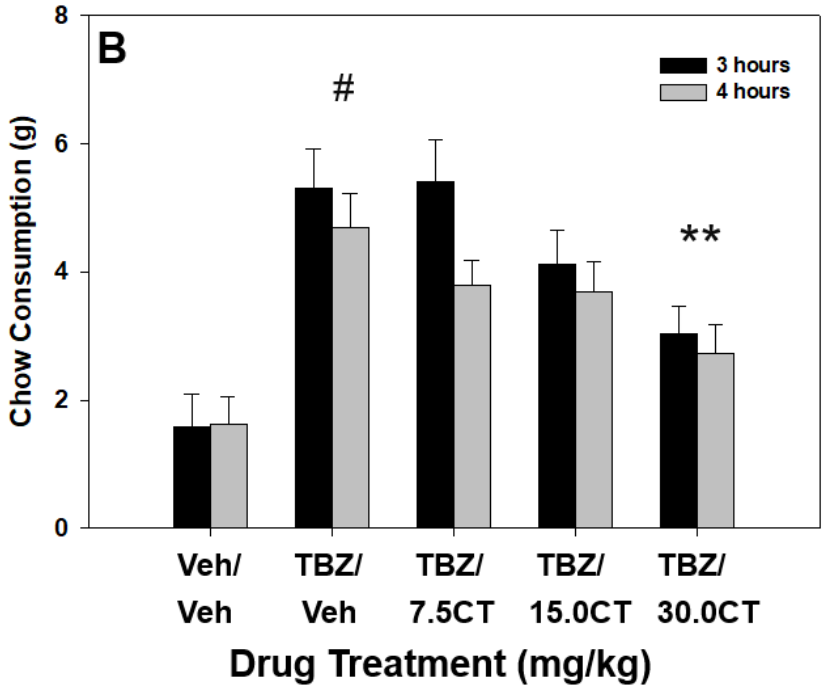



**Figure 5.2.** The effects of the DAT inhibitor CT-005404 on TBZ-induced changes in performance on the FR5 concurrent lever pressing/chow feeding choice procedure. **(A)** Lever presses during the 30-minute session. #$p$<0.001, TBZ plus vehicle significantly differed from vehicle plus vehicle; **$p$<0.01, TBZ plus 15.0 mg/kg CT-005404 (3 hours) significantly differed from TBZ plus vehicle; *$p$<0.05, TBZ plus 30.0 mg/kg CT-005404 significantly differed from TBZ plus vehicle. **(B)** Gram quantity of chow intake. #$p$<0.001, TBZ plus vehicle significantly differed from vehicle plus vehicle; **$p$<0.01, TBZ plus 30.0 mg/kg CT-005404 significantly differed from TBZ plus vehicle.

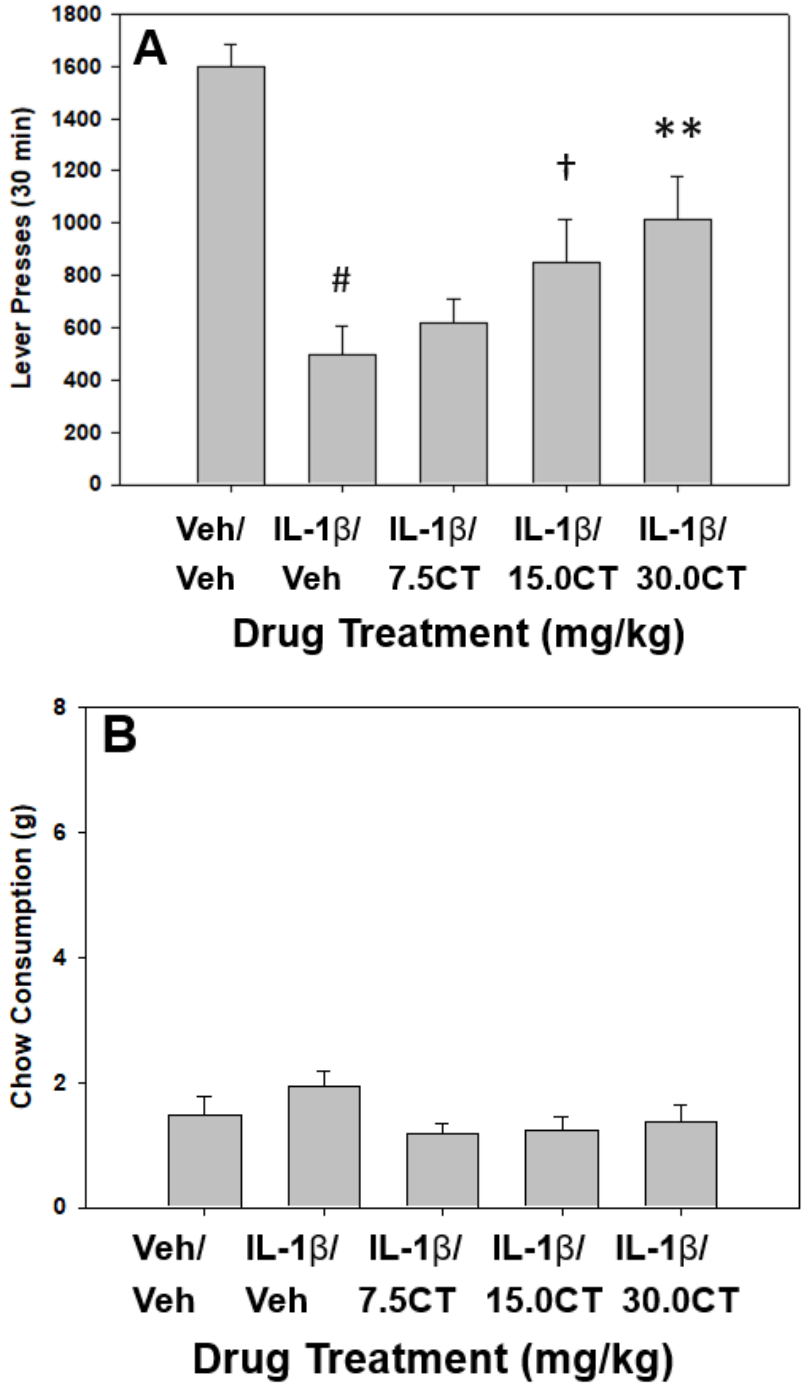
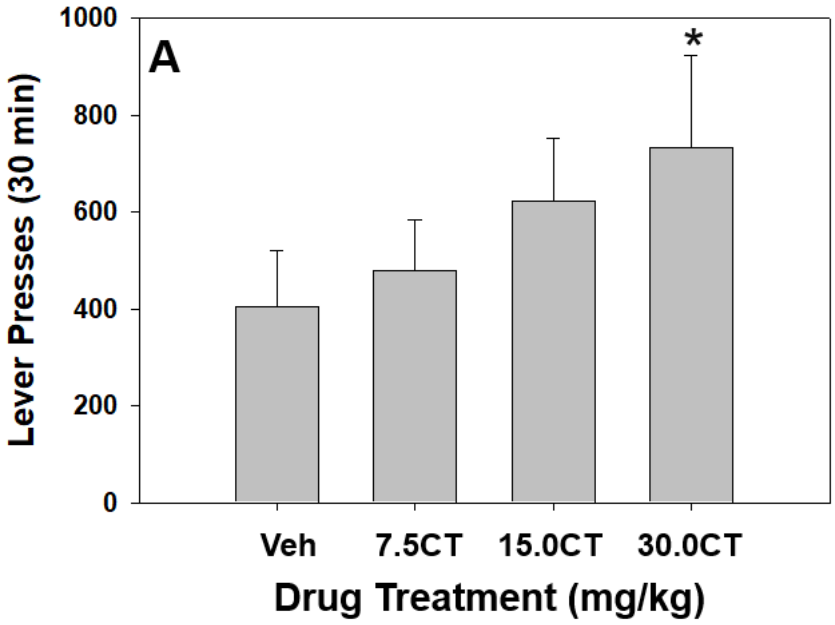



**Figure 5.3.** The effects of the DAT inhibitor CT-005404 on IL-1β-induced changes in performance on the FR5 concurrent lever pressing/chow-feeding choice procedure. **(A)** Lever presses during the 30-minute session. #$p<0.001$, IL-1β plus vehicle significantly differed from vehicle plus vehicle; † $0.10>p>0.05$, IL-1β plus 15.0 mg/kg CT-005404 trended towards a

significant difference from IL-1β plus vehicle; **p< 0.01, IL-1β plus 30.0 mg/kg CT-005404 significantly differed from IL-1β plus vehicle. **(B)** Gram quantity of chow intake. IL-1β plus vehicle did not significantly differ from vehicle plus vehicle.



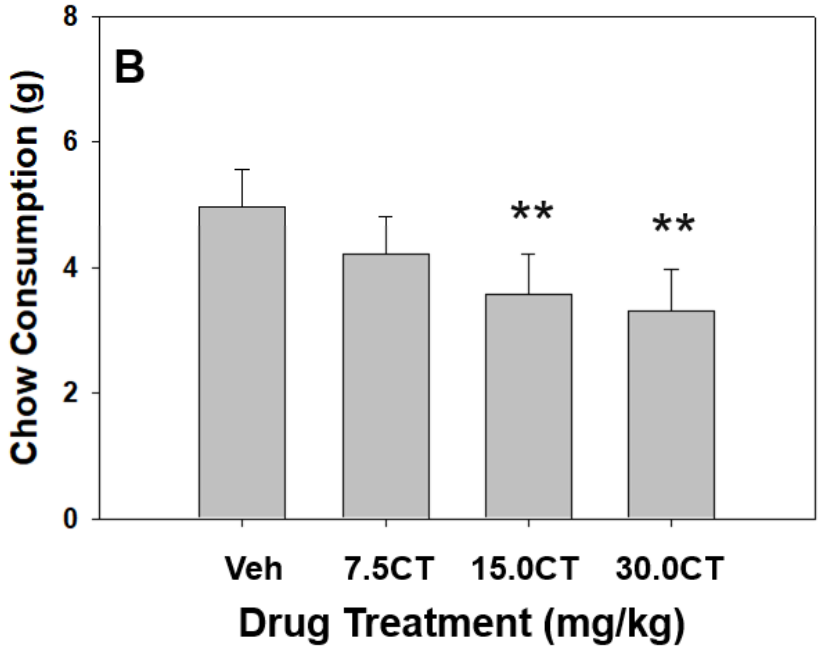



**Figure 5.4.** The effects of the DAT inhibitor CT-005404 on performance on the Progressive Ratio/chow-feeding choice procedure. **(A)** Lever presses during the 30 minute session. Rats treated with 30.0 mg/kg CT-005404 lever pressed significantly more than vehicle-treated rats (*$p<0.05$). **(B)** Gram quantity of chow intake. Rats treated with 15.0 mg/kg and 30.0 mg/kg CT-005404 consumed significantly less chow than rats treated with vehicle (**$p<0.01$).

# Chapter 6: Effort-related effects of altering lever pressing ratio requirements, and of tetrabenazine administration, on progressive ratio work output.

## 6.1 Introduction

Motivation has been defined as the set of processes through which organisms regulate the probability, proximity, and availability of significant stimuli (Salamone 1992; Salamone and Correa 2002; Salamone 2010; Salamone et al. 2016a). Motivated behaviors are characterized by a high degree of behavioral activation, which is manifested as the vigor and persistence of work output (Salamone 1988, 1992; Salamone and Correa 2002, 2012; Croxson et al. 2009; McGinty et al. 2013; Salamone et al. 2016a). These features facilitate the exertion of effort during the instigation and maintenance of instrumental responding, allowing organisms to overcome effort-related response costs in order to obtain something valued by the organism (e.g. food). Considerable research has shown that mesolimbic dopamine (DA) plays an important role in regulating motivated behaviors (Salamone et al. 1997, 2007, 2012; Salamone and Correa, 2002, 2012; Robbins and Everitt, 2007). More specifically, interference with nucleus accumbens DA can affect the willingness of animals to exert high levels of effort to obtain food reinforcers, while leaving primary aspects of motivation such as appetite and the direction and acquisition of food intact (Salamone and Correa 2002, 2012).

A growing body of clinical neuroscience research emphasizes the importance of effort-related symptoms, such as fatigue, anergia, and psychomotor retardation in major depression, multiple sclerosis, chronic fatigue syndromes, schizophrenia, Parkinson's disease, and other neurological and psychiatric disorders (Stahl 2002; Demyttenaere et al. 2005; Salamone et al.

2006; Treadway and Zald 2011; Fava et al. 2014; Chong et al. 2015). Human tests of effort-related decision making are increasingly being employed for characterizing the motivational impairments seen in people with Parkinsonism, schizophrenia, and depression (Treadway et al. 2012a; Gold et al. 2013, 2015; Chong et al. 2015; Green et al. 2015; Green and Horan 2015; Reddy et al. 2015). Patients with major depression have a reduced likelihood of selecting high effort alternatives when assessed in tests of effort-related decision making (Treadway et al. 2012a; Yang et al. 2014). Furthermore, Guillion and Rush (1998) conducted a factor analytic study of patients with major depression and identified a "lack of energy" factor (i.e., problems with energy/fatigability, psychomotor retardation, inability to work), which was the factor that loaded most strongly onto a second order general depression factor. These symptoms can strongly interfere with daily living, and can be difficult to treat (Tylee et al. 1999; Stahl 2002; Fava et al. 2014). Moreover, it has been suggested that fatigue could be used as a predictor of clinical depression, and can contribute to deficits in global functioning (Corfield et al. 2016), highlighting its importance as a critical feature in animal research. Thus, it is important to study the neural and behavioral processes related to the exertion of effort to better understand the physiological underpinnings of these symptoms.

In view of the clinical relevance of motivational dysfunctions related to deficits in behavioral activation and exertion of effort, it is critical to develop animal models that focus specifically on effort-related motivational symptoms. Animal studies of effort-based choice have been developed as formal models to study motivational dysfunctions. In these tasks, animals are offered a choice between high-effort instrumental actions leading to highly valued reinforcers, versus low-effort options leading to less valued reinforcers. Fixed ratio (FR) reinforcement schedules offer trained animals the option to exert high levels of responding for a preferred food

reinforcer (high-carbohydrate pellets), or to choose a concurrently available but less preferred option (standard laboratory chow). DA $D_1$ and $D_2$ family antagonists decrease food-reinforced FR5 lever pressing but substantially increase chow intake (Salamone et al., 1991, 2002; Sink et al. 2008). The shift from lever pressing to chow intake is not produced by neostriatal DA depletions or antagonism (Cousins et al. 1993; Farrar et al. 2010), but instead results from accumbens DA depletions, and intra-accumbens injections of $D_1$ or $D_2$ antagonists (Cousins et al. 1993; Nowend et al. 2001; Farrar et al. 2010). Drug treatments that produce the shift in choice behavior did not alter food intake or preference in free-feeding choice tests (Salamone et al. 1991; Koch et al. 2000; Farrar et al. 2008), and did not resemble the effects of appetite suppressant drugs (Salamone et al. 2002; Sink et al. 2008; Randall et al. 2012, 2013).

The Salamone lab also has employed a progressive ratio (PROG)/chow feeding concurrent choice task (Salamone 2006; Randall et al. 2012, 2014, 2015; Yohn et al. 2016b,c,d, 2018), which offers the choice of lever pressing on a PROG schedule reinforced by the preferred high carbohydrate pellets vs. approaching and consuming the less preferred chow. The PROG schedule requires that the rat repeatedly make within-session choices between lever pressing and chow intake under conditions in which the ratio requirement is gradually incrementing. DA $D_1$ and $D_2$ family antagonists decreased PROG lever pressing (e.g., number of lever presses, highest ratio achieved, and time spent responding), but rats maintained normal levels of chow intake, indicating that their appetite for food was still intact (Randall et al. 2012, 2014). Importantly, the effects of DA antagonism or depletion differed markedly from those of reinforcement or appetite-related manipulations (reinforcer devaluation by pre-feeding, the cannabinoid $CB_1$ antagonist/inverse agonists AM251 and AM4113), which decrease both lever pressing and chow intake (Randall et al. 2012, 2014). In summary, low-to moderate doses of DA antagonists, or

local antagonism or depletion of DA in nucleus accumbens, do not affect primary food motivation or appetite when access to food is unconstrained, but instead make it less likely that animals will work for food. These findings demonstrate that interference with DA transmission causes animals to reallocate their instrumental actions based on the response requirements of the task, and select lower cost alternatives to obtain food (Salamone et al. 2007, 2009b, 2012, 2016a,b,c; Salamone and Correa 2002, 2012).

Due to the prevalence of effort-related dysfunctions observed in human psychopathologies, it is critical to characterize the impact of varying response costs on an organism's willingness to exert high levels of effort. Human clinical research has shown that willingness to work for monetary reward on a PROG task was impaired in people with depression (Hershenberg et al. 2016), and in schizophrenics with high levels of negative symptoms (Wolf et al. 2014). PROG schedules can also be used to measure exertion of high-effort behavior in rats (Salamone 2006; Randall et al. 2012, 2014, 2015; Yohn et al. 2016b,c,d, 2018). Typically, animals tested on PROG schedules will lever press until the work requirement, or the 'cost', becomes too high, while others will continue responding throughout the entire session. The point at which an animal ceases high-effort responding is referred to as the breakpoint, and can be used as a measure of maximal effort exertion. In the concurrent PROG/chow feeding choice procedure described above, the freely available lab chow acts as a 'magnet', effectively pulling the animal away from high-effort lever pressing towards a low-effort alternative, as soon as the animal perceives the work requirement as too difficult to overcome. In the present experiment, rats were trained on a PROG reinforcement schedule without the option of freely available lab chow, so the only way to receive food was to lever press. In addition, the PROG schedule was manipulated to produce a steeper ratio increment

increase in order to shift animals' breakpoint earlier in order to be observed within a 30-minute session.

The present studies were conducted to evaluate the effects of altering ratio requirements on a PROG schedule of reinforcement, and to establish a PROG schedule that is difficult enough to cause animals to exhibit a breakpoint during a 30-minute session. It was predicted that altering the number of reinforcers delivered at each ratio (N), and the increment at which the ratio requirement increases after N reinforcers are delivered (I), would produce a drastic change to the total number of lever presses and breakpoint exhibited during a session. It also was hypothesized that the VMAT-2 inhibitor TBZ, which pharmacologically depletes DA, would decrease the number of lever presses, the time of last response, and the breakpoint during the modified PROG session. Ultimately, the PROG schedule determined by these experiments will be used in future electrophysiological recording experiments to assess frontal cortex electroencephalography (EEG) changes under baseline and pharmacological conditions in which animals shift their behavior away from high-effort instrumental responding towards the low effort-alternative (see Chapter 7 for initial development of recording techniques and effects of TBZ in untrained animals).

## 6.2 Materials and Methods

*Animals*

Sixteen adult male Sprague Dawley rats were obtained from Envigo (Indianapolis, IN, USA), and were housed in a colony maintained at 23 °C with 12-h light/dark cycles (lights on 07:00). Rats weighed 275–299 g at the beginning of the study, and were initially food restricted

to 85% of their free-feeding body weight for operant training. Rats were fed supplemental chow to maintain weight throughout the study, with water available ad libitum. Rats were allowed modest weight gain throughout the experiment. Animal protocols were approved by the University of Connecticut Animal Care and Use Committee, and followed NIH guidelines.

*PROG Lever Pressing Task and Schedule Modifications*

Rats were initially trained to lever press for 45 mg high-carbohydrate pellets (Bio-Serv) on an FR1 (continuous reinforcement) schedule for one week or until a stable baseline was reached. Then, rats were switched to a PROG schedule (Randall et al. 2012, 2014, 2015), in which the number (N) of pellets delivered at each ratio was equal to 15, and the increment (I) at which the ratio increased after the delivery of each reinforcer was equal to 1 (e.g. FR1 x 15 reinforcers, FR2 x 15 reinforcers, FR3 x 15 reinforcers, and so on) for nine weeks (30 min sessions, 5 times/week). The PROG schedule was then modified so that N = 1 and I = 1 (e.g. FR1 x 1 reinforcer, FR2 x 1 reinforcer, FR3 x 1 reinforcer, and so on), and rats were trained on this schedule for six and a half weeks. After a stable baseline was reached, the PROG schedule was further modified to change the ratio increase increment after each reinforcer delivery, so that N = 1 and I = 2 (e.g. FR1 x 1 reinforcer, FR3 x 1 reinforcer, FR5 x 1 reinforcer, and so on). After three days of training on the modified PROG schedule, a stable baseline was reached. Then, the schedule underwent a final modification, increasing the ratio increment to I = 4 (e.g. FR1 x 1 reinforcer, FR5 x 1 reinforcer, FR9 x 1 reinforcer, and so on). Rats remained on this training schedule for three weeks before drug testing began. At the end of each 30 minute session, rats were immediately removed from the chambers and number of lever presses, time of last response, and breakpoint ratio (i.e. last successfully completed ratio during the session) was

recorded for each rat. On baseline training and drug treatment days, rats consumed all of the operant pellets that were delivered during each session.

*Drug Treatments and Dose Selection*

Tetrabenazine *(*TBZ) (9,10-dimethoxy-3-(2-methylpropyl)-1,3,4,6,7, 11b hexahydrobenzo[a]quinolizin-2-one) was obtained from Tocris Bioscience (Ellisville, MO) and was dissolved in DMSO (20%), 0.9% saline (80%), and was titrated with microliter quantities of 1.0 N HCl until the solid drug was in solution at a pH of 4.0-4.5. The DMSO/saline solution was administered as the vehicle control. The dose of 1.0 mg/kg TBZ was selected based on extensive piloting in our laboratory.

*Behavioral Pharmacology Experiment*

Fifteen out of sixteen trained rats were used for the behavioral pharmacology experiment. One rat was excluded from the experiment due to an unstable baseline. On drug testing days, trained rats were administered either TBZ (1.0 mg/kg) or vehicle via intraperitoneal (IP) injections 120 minutes before testing. The experiment used a within-groups design, with half of the rats receiving a vehicle treatment on the first day of drug testing, while the other half received TBZ, and vice versa on the second drug testing day. The only PROG schedule used for the behavioral pharmacology experiment was the schedule where N = 1 and I = 4, which was the final stage of training.

*Statistical Analysis*

For the PROG training progression, differences in lever presses across time within each phase of training was analyzed using repeated measures ANOVA. For the behavioral pharmacology experiment, number of lever presses, time of last response, and breakpoint ratio were all analyzed using paired-samples t tests. Factorial ANOVA was used to determine an overall main effect of treatment on the three behavioral measures during the PROG session as a percentage of their baseline performance. A statistical program (SPSS, Version 25) was used to perform all analyses.

## 6.3 Results

Rats underwent a training progression on various schedules of PROG reinforcement over a time period of 20 weeks. Shown in **Figure 6.1**, a repeated measures ANOVA demonstrated a statistically significant main effect of week on average lever presses during the 'N=15; I=1' phase [$F(8,120)=69.626$, $p<0.001$] and a significant linear trend [$F(1,15)=147.635$, $p<0.001$]. There was no significant effect of week on lever presses in the second phase, 'N=1; I=1' (p=n.s.), or final phase, 'N=1; I=4' (p=n.s.) (**Fig. 6.1**).

TBZ was administered to rats trained on the modified PROG reinforcement schedule (N=1; I=4). The mean number of lever presses under vehicle conditions was 1853.2 ± 259.5. TBZ administration yielded an average of 982.3 ± 194.8 lever presses, equating to a 47% reduction compared to the vehicle-treated group. Paired-sample t test indicated a statistically significant difference in lever presses between the vehicle and TBZ treatment groups [$t(14)=5.231$, $p<0.001$] (**Fig. 6.2A**). Time of last response and end of session breakpoint ratios

were also compared. Paired-sample t test indicated that TBZ significantly reduced the time of last response during the session [$t(14)=4.588$, $p<0.001$] (**Fig. 6.2B**). There was also a significant reduction of breakpoint ratio as a result of TBZ administration, as demonstrated by a paired-sample t test [$t(14)=4.885$, $p<0.001$] (**Fig. 6.2C**). TBZ administration resulted in a 29-30% reduction of both time of last response and breakpoint ratio measures.

**Figure 6.3** shows the effects of TBZ on PROG lever presses, time of last response, and breakpoint ratio during a 30-minute session in rats as a percentage of baseline performance. Factorial ANOVA indicated an overall significant main effect of treatment [$F(1,42)=39.283$, $p<0.001$] and a non-significant interaction between treatment and any of the dependent variables (p=n.s.). After reaching a stable baseline in the final phase, average baseline values were calculated for lever presses, time of last response, and breakpoint ratio. Prior to the first day of drug testing, the average number of lever presses was 1752.1 ± 310.5, average time of last response was 1371.0 ± 93.0 seconds, and average breakpoint ratio was 111.3 ± 10.6. Prior to the second day of drug testing, the average number of lever presses was 1803.9 ± 255.7, average time of last response was 1324.8 ± 86.4 seconds, and average breakpoint ratio was 114.3 ± 8.4 (data not shown). Paired sample t tests indicated no statistically significant differences between any of the baseline measures from the first to second drug testing day. These averages were used to calculate % baseline values (**Fig. 6.3**).

## 6.4 Figures

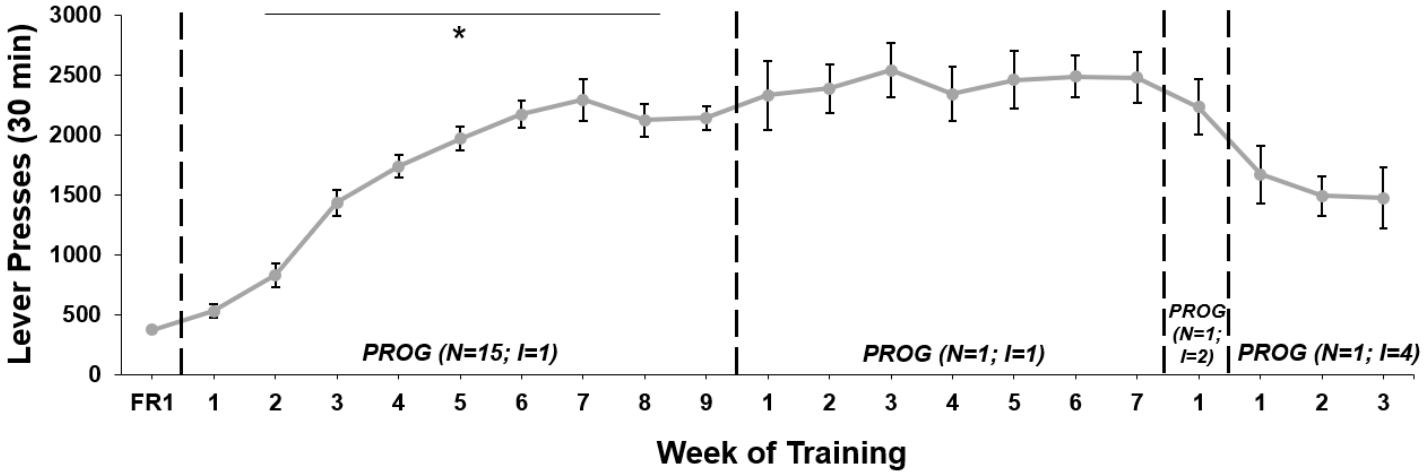
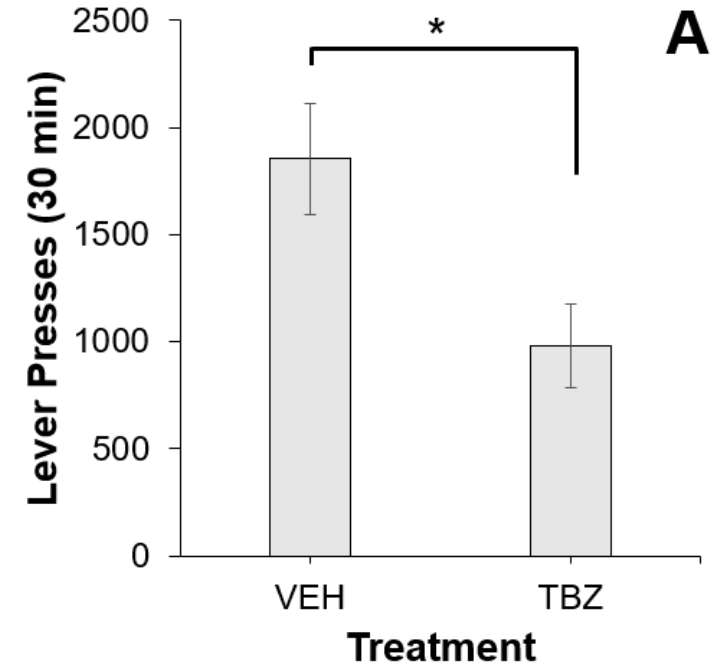
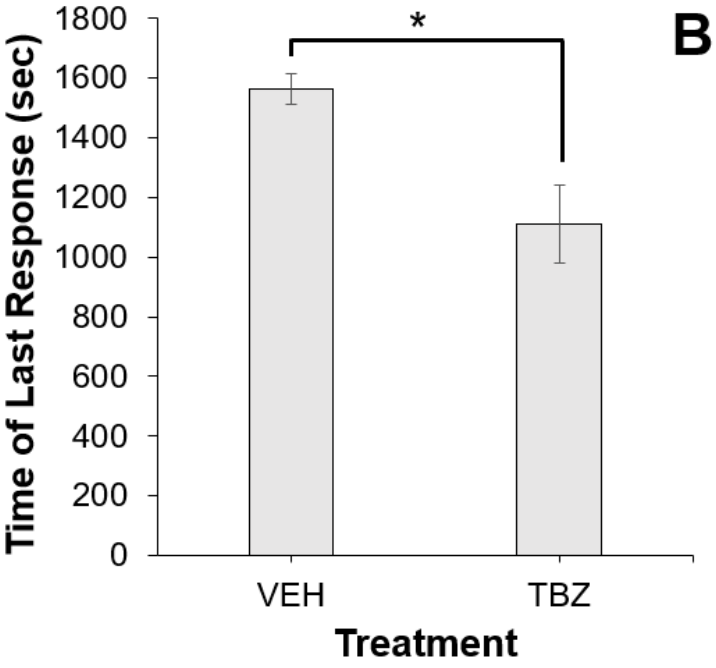



**Figure 6.1.** Progressive ratio (PROG) training procedure. Rats (n=16) were trained on various PROG schedules over the course of 20 weeks. Each point (●) represents weekly average lever presses ± SEM. Dashed lines represent training phase transitions, each phase labeled accordingly as 'PROG (N=X; I=X)'. Overall effect of week on lever presses during the first training phase (N=15; I=1) (*$p<0.001$).


A
*
2500
2000
1500
1000
500
0
Lever Presses (30 min)
VEH
TBZ
Treatment


B
*
1800
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600
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Time of Last Response (sec)
VEH
TBZ
Treatment

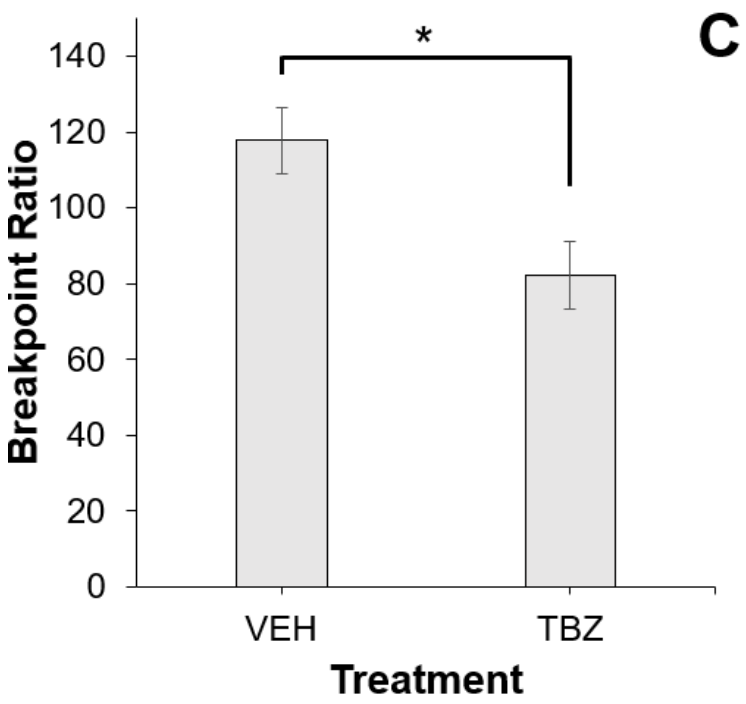



**Figure 6.2.** Effects of tetrabenazine (TBZ) on PROG lever presses, time of last response, and breakpoint ratio during a 30-minute session in rats (n=15). **(A)** Mean ± SEM number of lever presses after treatment with vehicle or 1 mg/kg TBZ. *p<0.001, difference between vehicle and TBZ. **(B)** Mean ± SEM time of last response (sec) in a 30-minute session after treatment with vehicle or 1 mg/kg TBZ. *p<0.001, difference between vehicle and TBZ. **(C)** Mean ± SEM breakpoint ratio in a 30-minute session after treatment with vehicle or 1 mg/kg TBZ. *p<0.001, difference between vehicle and TBZ.

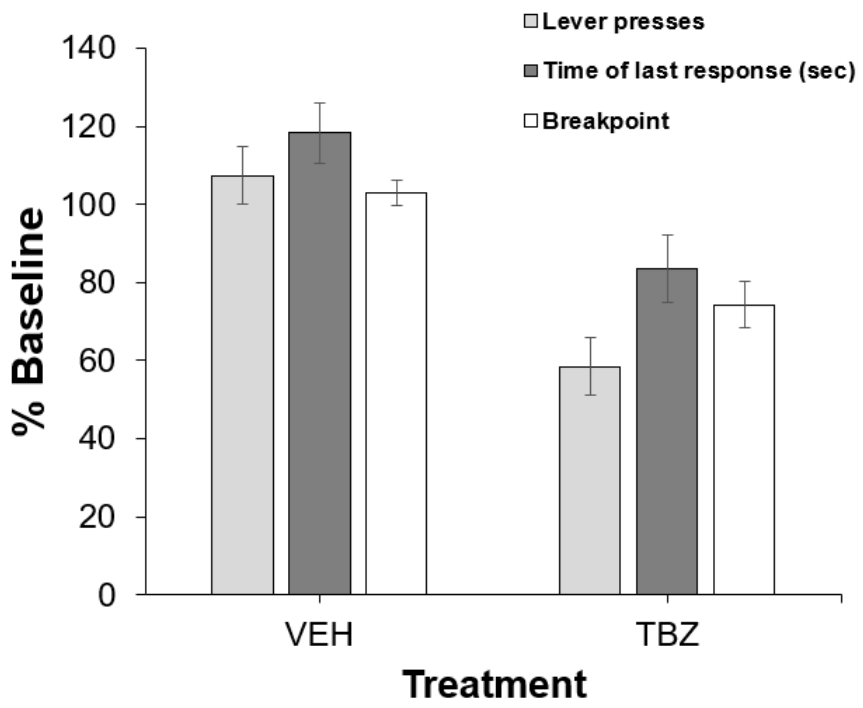



**Figure 6.3.** Effects of tetrabenazine (TBZ) on PROG lever presses, time of last response, and breakpoint ratio during a 30-minute session in rats (n=15) as a percentage of baseline performance. Factorial ANOVA indicated an overall significant main effect of treatment ($p<0.001$) and a non-significant interaction between treatment and any of the dependent variables (p=n.s.).

# Chapter 7: Exploration of frontal cortex electrophysiology in freely moving untrained rats: effects of TBZ.

## 7.1 Introduction

Motivational symptoms such as psychomotor slowing, fatigue, lassitude, loss of energy and reduced exertion of effort are critical and debilitating features of psychopathology (Stahl 2002; Demyttenaere et al. 2005; Salamone et al. 2006; Treadway and Zald 2011; Treadway et al. 2012a; Fava et al. 2014). Effort-related motivational symptoms are present in major depressive disorder, bipolar disorder, schizophrenia, Parkinsonism, chronic fatigue syndrome and multiple sclerosis (Salamone et al. 2006, 2010, 2016b; Friedman et al. 2007; Tellez et al. 2008; Green et al. 2015). The neural basis of the effort-related dysfunctions in psychopathology are still being characterized, nevertheless, considerable evidence shows that the selection of high-effort activities is dependent upon forebrain mesolimbic DA circuitry as well as integrity of the circuitry connecting ventral striatum, ventral pallidum, and prefrontal cortex (Salamone et al. 1991, 1994; 2007, 2016a,b, 2018; Walton et al. 2003; Floresco and Ghods-Sharifi 2007; Mingote et al. 2008; Farrar et al. 2008, 2010; Winstanley and Floresco 2016).

Our laboratory has demonstrated that tests of effort-based choice can be used as animal models of effort-related motivational symptoms in psychopathology (Salamone and Correa 2002; Salamone et al. 2007, 2016a,b,c, 2018). Pharmacological conditions that induce a low-effort bias in humans can alter effort-related choice in rats, and bias animals towards low-effort options (Salamone et al. 2016a,b,c; Yohn et al. 2016a,b,c). For example, rats treated with vesicular monoamine transport type-2 inhibitor tetrabenazine (TBZ), which induces or exacerbates anergia and fatigue symptoms in humans, can alter effort-related choice, reducing selection of the high

effort alternative. These effects can be reversed by co-administration of the established antidepressant bupropion (Wellbutrin), which inhibits catecholamine uptake, and the DA transport (DAT) blockers GBR12909, PRX-14040, lisdexamfetamine, methylphenidate, and modafinil. This pattern of effects is consistent with data from human clinical neuroscience on effort-related motivational dysfunctions in psychopathology, and the effects of drugs that act on DA. Though the neurochemical changes underlying these effects have been characterized through the use of animal studies, electrophysiological markers of the selection of high or low effort activity have not been defined. Moreover, it is unclear if such markers developed from animal studies can be readily translatable to human psychopathologies.

Recent evidence has indicated that there are electroencephalographic (EEG) markers of frontal cortex activity that are characteristic of engagement in motivated behavior and anticipation of reinforcement, and that these markers are reduced in depressed people (Nelson et al. 2018; Gheza et al. 2019). These findings are consistent with well characterized role of frontal cortex, including prefrontal areas, in regulating decision making processes (Winstanley and Floresco 2016). Preclinical tests using rodent effort-based choice tasks can serve as valuable models of motivational dysfunction in humans, since human tests of effort-related decision making derived from animal studies are increasingly being employed for characterizing the motivational impairments seen in people with Parkinsonism, schizophrenia, and depression (Treadway et al. 2012a; Gold et al. 2013, 2015; Chong et al. 2015; Green et al. 2015; Green and Horan 2015; Reddy et al. 2015). Patients with major depression have a reduced likelihood of selecting high effort alternatives when assessed in tests of effort-related decision making (Treadway et al. 2012a; Yang et al. 2014). Such translational results clearly validate the use of

tests of effort-based decision making as animal models of the effort-related symptoms in psychopathology.

A number of imaging papers have aimed to identify the neural correlates of psychomotor retardation, anergia, and lassitude in depression and other disorders (Hickie et al. 1999; Capuron et al. 2007), and several recent studies have reported on the brain areas involved in effort-related decision making (Wardle et al. 2011; Schouppe et al. 2014; Huang et al. 2016; Hogan et al. 2018; Aridan et al. 2019). Generally speaking, these studies have implicated striatal and frontal cortical processes in approach motivation and effort-based decision making, which is consistent with the animal studies reviewed above. Additional lines of inquiry have employed EEG methods to characterize alterations in approach motivation. One psychophysiological marker of instrumental approach motivation in humans is an asymmetry in EEG activity between left and right frontal brain regions (Allen, Coan, & Nazarian, 2004; Coan & Allen, 2004). Pizzagalli et al. (2005) observed that higher task-independent alpha EEG activity in left dorsolateral prefrontal and medial orbitofrontal cortices was associated with a stronger tendency to respond to reward-related cues, supporting the idea that frontal EEG asymmetry modulates the propensity to engage in appetitively motivated behavior. Moreover, several papers have reported that people with major depressive disorder, as well as individuals with high levels of depressive symptoms, exhibit decreased relative left frontal cortical activity at rest (Bruder et al., 1997; Henriques & Davidson, 1991; Diego, Field, & Hernandez-Reif, 2001; Feng et al., 2012; see Thibodeau, Jorgensen, & Kim, 2006 for a meta-analysis). A recent study by Nelson et al. (2018) investigated the association between multiple depression symptom dimensions and asymmetrical frontal cortical activity in people with major depression as well as two different control groups. EEG activity in frontal cortex was measured while people were performing a computerized slot

machine task, during which participants anticipated possible monetary reward in some conditions, or no reward in others. In undergraduates with low depression symptoms and control participants that had no history of depression, reward trials induced greater relative left frontal cortical activity relative to no incentive trials. Furthermore, in both samples across all participants, increased presentation of lassitude symptoms was associated with decreased relative left frontal cortical activity while anticipating reward. The authors concluded that *"depression symptoms consistent with motivational disengagement are associated with decreased relative left frontal cortical activity"* (Nelson et al. 2018). These results are consistent with a recent report by Gheza et al. (2019), who recorded frontal EEG activity from a large cohort of depressed patients that were performing a reinforcement learning task. Although depressed participants showed intact reinforcement learning compared to control participants, they had greater alpha EEG power asymmetry, consistent with the results of Nelson et al. (2018) and potentially reflecting lower approach motivation.

Although the precise relationship between frontal EEG in rats and humans is uncertain, the goal of this experiment was to characterize frontal and parietal cortex EEG activity from both left and right hemispheres in awake rats under baseline conditions, and then under pharmacologically-induced DA depletion conditions by administration of tetrabenazine (TBZ). This experiment was intended to be the first step in a larger project focusing on the development of physiological markers associated with altered DA transmission and effort-related dysfunction in preclinical animal models that can be readily translated to human studies. It was hypothesized that frontal cortex activity will provide a correlate of the pharmacological manipulations that affect the functions of ventral striatum and striatopallidal circuits that are known to be involved in effort-based decision making. Beyond the scope of the present studies, it is hypothesized that

frontal EEG markers can be developed that also are sensitive to the selection of high and low effort instrumental activities. Frontal cortex was selected as a region of interest because of the extensive animal and human literatures linking prefrontal cortex to executive function, decision making, and effort-based choice. Parietal cortex recordings were conducted to provide a comparison with the frontal recordings, and also to determine if other cortical areas are responsive to TBZ.

## 7.2 Materials and Methods

*Surgeries*

One cohort of untrained male (n=8) rats were used for the initial EEG recordings. Rats were anesthetized with an intraperitoneal injection of 100.0 mg/kg ketamine hydrochloride and 10.0 mg/kg xylazine prior to placement in a stereotaxic device. Up to six stainless-steel skull screw EEG electrodes were implanted adjacent to the frontal and parietal cortices, and as ground and reference electrodes. Rats were given seven days to recovery from surgery before recording took place.

*Electrophysiological Recording Experiment*

A Digital Lynx SX Electrophysiology System (Neuralynx) was used to acquire EEG activity from awake rats. Rats were connected to the electrophysiology system using a tethered cable. Wide-band activity (1-2000 Hz, 4006 samples/sec) will be recorded using the Neuralynx system and analyzed offline using MATLAB software (MathWorks Inc, Natick, MA). After a brief acclimation period, ~1 minute of baseline EEG activity was recorded from each rat. Then,

rats were injected with vehicle or TBZ (1.0 mg/kg). EEG activity was recorded immediately after injection for up to five minutes. A lead time of 120 minutes elapsed before resuming recordings. Each rat was exposed to vehicle and TBZ treatments, counterbalanced, over the course of two separate testing days (one treatment per week).

*Data Preprocessing*

All signal processing and related procedures were performed using the FieldTrip software package (Oostenveld et al. 2011; http://www.fieldtriptoolbox.org/) and custom scripts in MATLAB (The MathWorks, Natick, MA). EEG data were preprocessed as follows: 1) data were down-sampled from 32 kHz to 1 kHz; 2) data were mean subtracted; 3) data were split into 1-s epochs to remove the epochs with artifacts; and 4) the first epoch and the last epoch were removed, and the epochs that were visually inspected to contain high-amplitude artifacts were removed.

*Neurophysiological Analysis*

To assess how the power spectral density (PSD) of each frequency varied across time, a time frequency analysis was conducted using short-time Fourier transform. We segmented the preprocessed EEG data into 10-s epochs with 5-s overlap. Each epoch is windowed with a Hamming window. We estimated the PSDs over frequencies from 1 Hz to 50 Hz (0.5 Hz resolution).

For power spectral analysis, the preprocessed EEG data were segmented into 1-s epochs with 0.5-s overlap. We estimated the PSDs using Welch's overlapped averaged periodogram method (Welch, 1967) with a Hamming window on each epoch. We estimated the PSDs over frequencies from 1 Hz to 50 Hz (0.5 Hz resolution).

In addition to power in specific sub-bands, EEG signals throughout frontal cortex were also analyzed to assess coherence and phase relationships. Coherence values (see Bullock et al, 1990) for each channel pair will be computed using the Welch periodogram estimation procedure with a spectral resolution of ~ 2 Hz. Coherence is a measure of the linear association between two signals as a function of frequency. In this experiment, the consistency of the relative amplitude and phase between interhemispheric channels at each frequency was quantified. To ensure that the measured coherence values were not due to chance alone, a significance estimation procedure was used where the coherence estimate is compared to that of signals with identical magnitude spectrum but with zero phase coherence. For each channel pair, the cumulative distribution of the frequency-specific coherence values is created by circularly phase-shifting one signal in the pair randomly, calculating the coherence and bootstrapping the procedure 250 times (Efron and Tibshirani, 1993). This procedure guarantees that the signal spectrums are identical but have no linear association (because the phase information is removed). Coherence values for each pair were then analyzed using univariate analysis of variance (ANOVA) followed by Tukey tests.

The preprocessed EEG data were segmented into 1-s epochs with 0.5-s overlap. We estimated the coherence using Welch's overlapped averaged periodogram method (Welch, 1967) with a Hamming window on each epoch. The interhemispheric coherence was calculated using

the following formula, where P_xx and P_yy are the estimated power spectral densities of the two signals, and P_xy is the cross power spectral density of the two signals.

$$C_{xy} = \frac{[P_{xy}]^2}{P_{xx} \cdot P_{yy}}$$

We estimated the coherences over frequencies from 1 Hz to 50 Hz (0.5 Hz resolution).

### 7.3 Results

For the recording experiments, a power spectral analysis was performed to quantify the effects of TBZ (1.0 mg/kg IP) or vehicle treatment on EEG activity recorded from awake rats (n=6) (**Fig. 7.1**). At baseline, EEG spectral analysis showed the highest peak PSD at 7.5 Hz. This effect was detectable in frontal and parietal channels, but was more robust in the parietal channels. In general, TBZ caused a reduction in EEG power in a frequency range of 7-8 Hz in all four channels relative to baseline (**Fig. 7.1B**). Also, PSD was slightly reduced at higher frequencies (e.g. >25 Hz in frontal channels; >35 Hz in parietal channels) in the TBZ condition compared to vehicle.

Analyses of inter-hemispheric EEG signal coherence revealed much stronger coherence measurements from electrodes that were placed in the same position on either side of the midline (e.g. left and right frontal electrodes) compared to electrodes that were placed in different positions on either side of the midline (e.g. left frontal and right parietal electrodes). Specifically, on a scale of 0-1, coherence was greater than 0.5 for congruous electrode placements at baseline under both vehicle (**Fig. 7.2A**) and TBZ (**Fig. 7.2B**) conditions, while coherence for incongruous

electrode placements remained below 0.5 in both treatment conditions across frequencies. Data from one rat was removed from the coherence analyses due to missing signal from one electrode.

### 7.4 Figures

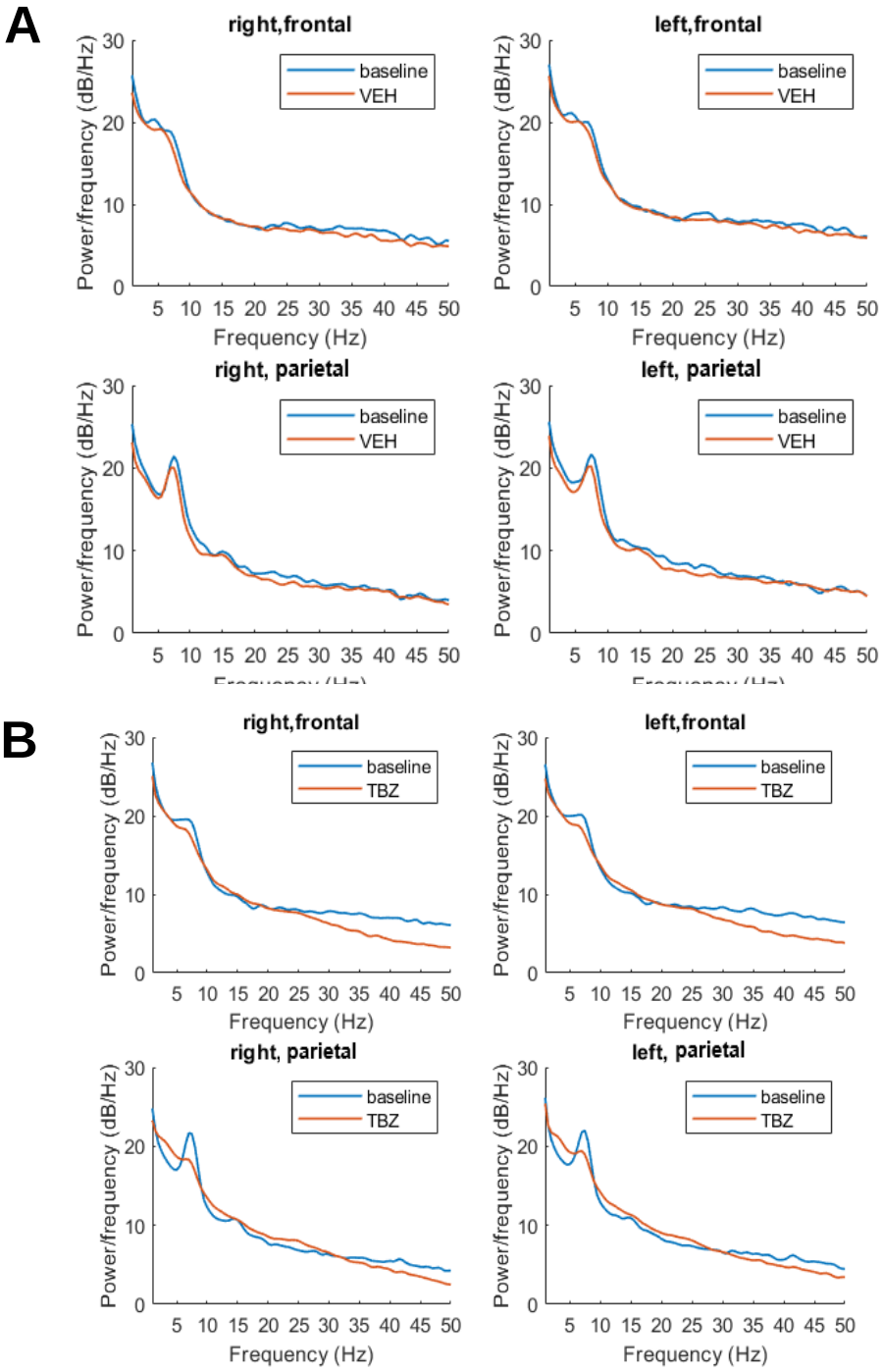


**Figure 7.1.** Effects of TBZ on power spectral density (PSD) (dB/Hz) of frequency (Hz) measured by EEG in left and right frontal and parietal electrodes. **(A)** PSD of EEG signal under baseline and vehicle conditions. **(B)** PSD of EEG signal under baseline and TBZ conditions.

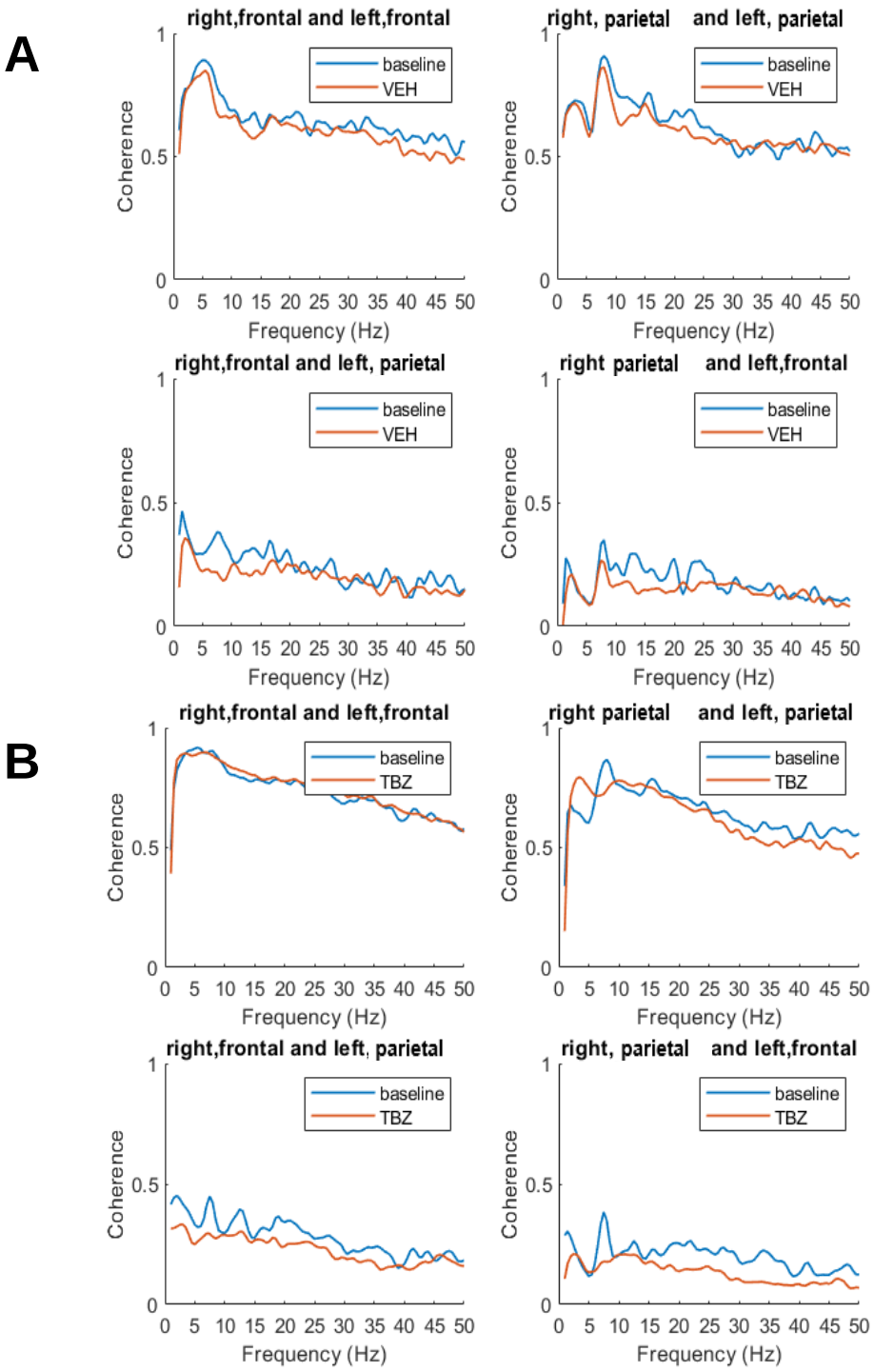



**Figure 7.2.** Effects of TBZ on inter-hemispheric coherence by frequency (Hz) measured by EEG in left and right frontal and parietal electrodes. **(A)** Coherence analysis of EEG signal under baseline and vehicle conditions. **(B)** Coherence analysis of EEG signal under baseline and TBZ conditions.

## Chapter 8: Discussion

### 8.1 Summary of Results

*The major objectives of the present research outlined in this dissertation were to investigate the role of DA in a model of effort-related motivational dysfunction in rats, to explore novel treatment options for motivational symptoms of depression and other disorders through the use of effort-based choice paradigms, and to identify physiological biomarkers related to motivational dysfunctions.*

The first set of experiments, outlined in **Chapter 2**, assessed the effects of chronic subcutaneous administration of the DA $D_2$ antagonist haloperidol on effort-based choice. This specific procedure was used to mimic the development of secondary negative symptoms which can be induced by long-term drug treatment. The results showed that chronic haloperidol administration significantly and dose-dependently reduced lever presses and increased chow intake during the FR5/chow feeding choice task and that continuous infusion of haloperidol via subdermal pumps provided a translatable model of chronic slow-release DA $D_2$ antagonist administration.

The experiments described in **Chapters 3 and 4** provided an initial characterization of the effort-related behavioral effects of the novel atypical DAT inhibitors that are analogs of modafinil, ***(S)***-CE-123 and *(S, S)*-CE-158. Co-administration of 24.0 mg/kg ***(S)***-CE-123 (**Chapter 3**) with TBZ produced a significant but partial reversal of the effects of TBZ and significantly increased PROG lever pressing, accompanied by a decrease chow intake. Furthermore, the effective dose of ***(S)***-CE-123 in both the TBZ reversal and PROG/chow feeding choice studies significantly increased extracellular DA in nucleus accumbens core as measured

by microdialysis. The experiments in **Chapter 4** demonstrated the ability of the modafinil analog *(S, S)*-CE-158 to reverse the effort-related effects of TBZ, increase PROG/chow lever pressing, and decrease chow intake during PROG/chow sessions. In addition, systemic administration of *(S, S)*-CE-158 increased extracellular DA in the nucleus accumbens during the behaviorally active time course. Ultimately, this research will guide future studies to advance target identification for treatments of motivational dysfunction.

The experiments discussed in **Chapter 5** provided a characterization of the effort-related effects of another recently synthesized DAT inhibitor known as CT-005404. This compound was assessed for its ability to reverse the effort-related effects of TBZ, and the suppression of lever pressing induced by the pro-inflammatory cytokine IL-1β. CT-005404 also produced a significant increase in lever pressing and decrease in chow intake when administered to rats on the PROG/chow procedure, and significantly increased extracellular accumbens DA over the course of >340 minutes. Collectively, results from **Chapters 3-5** suggest that novel atypical DAT inhibitors such as ***(S)***-CE-123, ***(S, S)***-CE-158, and CT-005404 may ultimately be useful as compounds that are suitable for treating motivational dysfunction.

A critical goal of the final experiments in this dissertation (**Chapters 6-7**) was to employ a novel operant lever pressing task and EEG methods to aid in the development of physiological markers associated with altered DA transmission and effort-related dysfunction in preclinical animal models that can be readily translated to human studies. In **Chapter 6** experiments, the PROG operant reinforcement schedule was modified to increase the overall work requirement. After achieving a steady baseline level of responses on the modified PROG schedule, TBZ was administered, which significantly reduced number of lever presses, time of last response in the session, and breakpoint ratio. This information was used to guide the experimental design

outlined in **Chapter 7**. The final experiments (**Chapter 7**) employed frontal cortex skull screw EEG activity recordings in behaving rats under baseline and pharmacological DA manipulations. Results from this experiment suggested several notable differences in relative frequency band power and inter-hemispheric coherence as a result of TBZ administration, which can be used as an initial step towards identifying a physiological biomarker of a low-effort bias and motivational dysfunctions as seen in clinical depression.

**8.2 Chapter 2 Discussion:**

The experiments described in **Chapter 2** were conducted to evaluate the effects of chronic DA $D_2$ antagonism in an animal model of effort-related choice behavior in rats. A chronic subcutaneous infusion of either vehicle, a high dose (HAL 2.0 mg/ml, 3.0 µl/hour), or a low dose (HAL 1.0 mg/ml, 3.0 µl/hour) of haloperidol was delivered via minipump for a period of four weeks, followed by a four-week washout. Consistent with previous work involving acute haloperidol administration (Salamone et al. 1991, 2009; Yohn et al. 2017), our results demonstrated that continuous chronic infusions of haloperidol to rats produced significant reductions of high-effort lever pressing on the FR5 procedure (**Fig. 2.1**), and a shift from lever pressing to chow intake on the concurrent FR5/chow feeding choice task (**Fig. 2.2**).

The effects of haloperidol in rats performing on the FR5 lever pressing schedule are depicted in **Figure 2.1**. In the drug exposure phase, significant overall effects of week and treatment group were found. Across weeks, drug treatment produced a significant overall effect on FR5 lever pressing, which was evident when the high dose was compared to vehicle, but not when the low dose was compared to vehicle, demonstrating that only the high dose rats lever pressed significantly less than the vehicle group throughout the drug exposure phase. There was

very little carry over of the drug effect into the washout phase, as demonstrated by a significant reduction in lever presses in the high dose group relative to vehicle at only at week 1. This effect was completely washed out by the second week. During the washout phase of Exp 2.1, a significant effect of week and week x treatment interaction were found, with no overall significant effect of treatment group, as the average number of lever presses returned to baseline.

Haloperidol produced a different pattern of dose-related effects on lever pressing in Exp 2.2. As shown in **Figures 2.1 and 2.2A**, rats in the high dose groups in both studies showed substantial and significant reductions in lever pressing, which were marked by very large effect sizes. However, the effects of the low dose were very different in the two studies. While the low dose did not significantly suppress lever pressing when the FR5 schedule was the only path to obtain food, and the effect size was relatively low (0.372), there was a significant suppression of lever pressing in the low dose group that was seen when animals were tested on the FR5/chow feeding choice task, and the effect size was much larger (0.744). This difference can be attributed to having the option of freely available lab chow offered in the FR5/chow feeding choice task. When a substitute food source was available (FR5/chow, Exp 2.2), rats treated with the low as well as the high dose of haloperidol shifted away from FR5 lever pressing and towards consumption of the freely available lab chow. However, when the only way to obtain the food reinforcer was to press the lever (FR5 alone, Exp 2.1), the low dose of haloperidol did not significantly reduce the tendency of rats to engage in FR5 lever pressing. These findings are consistent with the results of Cousins and Salamone (1994), who compared the effects of nucleus accumbens DA depletions induced by 6-hydroxydopamine on FR5 lever pressing vs. performance on the FR5/chow feeding choice task. They reported that while moderate depletions of accumbens DA shifted choice behavior in rats performing on the FR5/chow feeding choice

task, decreasing lever pressing and increasing chow intake, the same animals showed no significant suppression of FR5 responding when no concurrent chow was available. Similar findings were described by Yohn et al. (2015a), in which the DA depleting agent TBZ was assessed for its effort-related effects on a T-maze barrier choice task in rats. TBZ reduced selection of the high-effort behavior when there was a low-effort option, but did not affect high density vs. low density arm selection when there was no barrier, or when there were only pellets in one arm. Such an effect on T-maze performance was also seen after administration of haloperidol and nucleus accumbens DA depletions induced by 6-hydroxydopamine (Salamone et al. 1994). In mice tested on the T-maze barrier choice task, haloperidol reduced selection of the arm with the high density of food reinforcement when that arm was blocked by a barrier, but when both arms had a barrier, haloperidol did not affect choice (Pardo et al. 2012). Taken together, these results indicate that the effects of haloperidol and accumbens DA depletions are not simply due to an effect on primary food reinforcement, appetite, or discrimination of reinforcement magnitude. In this regard, it is worth emphasizing the effects of DA antagonism via chronic infusion of haloperidol in this experiment produced effects that do not resemble the effects of reinforcement or appetite-related manipulations (reinforcer devaluation by pre-feeding, administration of cannabinoid $CB_1$ antagonist/inverse agonists) in rats (Randall et al. 2012, 2014), or in mice (Pardo et al. 2012; Yang et al. 2020), which decreased both lever pressing and chow intake. Nevertheless, instrumental responding is consistently impaired by manipulations that interfere with DA transmission when either there is a very high effort-related challenge (e.g. Aberman and Salamone 1999; Salamone et al. 2001) or when animals have a low-cost alternative as a substitute (i.e., the effort-based choice tasks).

After prolonged exposure to a drug, changes in the effects of the drug, termed 'tolerance' or 'sensitization,' can occur due to physiological or psychological factors. These phenomena are often difficult to measure in humans due to extreme variability in an individual's previous drug exposure and medication compliance, genetic factors, comorbidities, concomitant treatment regimens, etc., and are often overlooked by clinicians (Li 2016). Animal research has suggested that the frequency of repeated antipsychotic dosing (e.g. intermittent vs. continuous) can influence a drug's behavioral effects, including potential tolerance and sensitization (Kurachi et al. 1995; Trevitt et al. 1998; Gao and Li 2014; Sebel et al. 2017). The present experiments were designed to assess the effects of continuous exposure to haloperidol at two different doses over the course of four weeks, and to observe for signs of tolerance or sensitization, demonstrated by either an increase or decrease in the effects of the drug across weeks. In these experiments, there was clearly an overall significant effect of haloperidol treatment, but the lack of a week x treatment interaction indicates that rats did not develop tolerance or sensitization to haloperidol at either dose. Nevertheless, the response to acute haloperidol after washout from a period of chronic administration demands further exploration in order to elucidate the possible roles of drug exposure duration and concentration on the development of tolerance.

In addition to effects such as drug tolerance or sensitization, prolonged drug exposure can produce other undesirable effects. Previous work has demonstrated that repeated daily dosing of haloperidol over the course of 14 days significantly reduced lever pressing and increased chow intake, without producing a sedative (i.e., drowsiness) effect in the dose range tested (0.05-0.15 mg/kg) (Salamone et al. 1996). In contrast, the atypical antipsychotic drug clozapine clearly produced sedation (i.e. drowsiness and sleepiness as observed with a reliable rating scale), and this effect was clearly related to the suppression of lever pressing induced by clozapine. Thus,

while some antipsychotic drugs show a dissociation between sedative effects and the suppression of lever pressing, with other drugs these actions appear to be related to each other. The present studies did not assess sedation or reinforcer preference in the absence of a work component, but our results suggest that possible sedative effects were unlikely, due to the very substantial intake of chow consumption observed during the concurrent FR5/chow feeding choice task in Exp 2.2. This effect was steady over the course of four week drug exposure phase (**see Fig. 2.2**), and no overt behavioral differences or drowsiness were observed. Moreover, animals in this study were able to maintain their target weights throughout the experiment (supplemental chow was given as needed), whereas rats in a previous pilot experiment given a higher concentration of haloperidol (3.0 mg/ml) were unable to maintain target weights. Therefore, a 'high dose' of 2.0 mg/ml was determined to be effective at suppressing lever pressing and increasing chow consumption by altering effort-related choice behavior without any apparent sedation. Moreover, the low dose was even more selective, in that it clearly suppressed lever pressing and shifted animals to chow intake when there was a choice, but failed to significantly suppress lever pressing when the only way to obtain food was to press the lever (i.e., FR5 alone).

This work has important implications for advances in clinical drug therapies for schizophrenia and related disorders. At baseline, individuals with schizophrenia exhibit effort-related impairments and other negative symptoms (Gold et al. 2013; Strauss et al. 2014; Treadway et al. 2015). As described earlier, some of the most common antipsychotics do not treat these symptoms, and may even induce or exacerbate them. The present findings highlight the effort-related motivational impairments that can be produced by antipsychotic medications such as haloperidol. Several types of drugs such as adenosine $A_{2A}$ receptor antagonists, glycine uptake inhibitors, and atypical DAT inhibitors have demonstrated the ability to attenuate the

motivational effects of DA $D_2$ antagonists and DA depleting agents in animals with limited signs of potential side effects (Salamone et al. 2009; Yohn et al. 2017; Rotolo et al. 2019), however, these potential treatments have not yet been assessed in chronic administration models. Future studies should assess whether chronic co-administration of drugs in these classes reduces the effects of reduced DA transmission, which may ultimately improve clinical therapeutic strategies for the negative symptoms of schizophrenia and other affective disorders.

### 8.3 Chapter 3 Discussion:

The synthesis of ***(S)***-CE-123 afforded highly pure desired enantiomer, and NMRs and mass spectroscopy methods yielded spectra unambiguously identifying the compound (see supplementary materials of Rotolo et al. 2019). In previous studies, the absolute configuration was characterized as the ***(S)***-enantiomer which was shown to be stronger at inhibiting DAT than the ***(R)***-enantiomer (Nikiforuk et al. 2017). In uptake inhibition assays conducted in HEK293 cells stably expressing human isoforms of DAT, NET and SERT, the $EC_{50}$ for inhibition of DAT by ***(S)***-CE-123 was reported to be $2.76 \times 10^{-6}$M, which was 30-fold selective relative to inhibition of NET and more than 400-fold selective compared to SERT inhibition (Nikiforuk et al. 2017, supplementary figure 17A). Thus, we present the first enantioselective synthesis of ***(S)***-CE-123.

The behavioral studies sought to provide an initial characterization of the behavioral effects of the novel atypical DAT inhibitor, ***(S)***-CE-123, in terms of its ability to alter effort-related choice behavior in rats. Specifically, one of the experiments employed a task designed to measure effort-related choice, which was used to assess the ability of ***(S)***-CE-123 to reverse the

effort-related effects of TBZ. In this experiment, rats trained on the FR5/chow feeding choice task shifted from the high effort option (FR5 lever pressing) to the low-effort option (chow intake) when treated with TBZ, consistent with previous findings (Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2015a,b,c). Several previous studies with TBZ have shown that these shifts in effort-based choice are not due to changes in food intake, food preference, sucrose preference, motor incapacity, or reference memory, and do not resemble the effects of appetite suppressant drugs or reward devaluation by pre-feeding (Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2015a; Pardo et al. 2015). Co-administration of 24.0 mg/kg ***(S)***-CE-123 with TBZ produced a significant but partial reversal of the effects of TBZ, as indicated by an increased selection of FR5 lever pressing (**Fig. 3.2A**) and a decrease in chow intake (**Fig. 3.2B**) compared to TBZ plus vehicle. In a parallel control experiment in rats tested on the same task, 24.0 mg/kg ***(S)***-CE-123 administered in the absence of TBZ did not did not have any effect on lever pressing (**Fig. 3.3A**) or chow intake (**Fig. 3.3B**).

In order to develop a more comprehensive characterization of the effort-related actions of monoamine uptake inhibitors, it also is important to administer them alone, in the absence of a drug like TBZ, because that would allow for the assessment of both increases and decreases in performance, in the absence of a drug condition like TBZ, which induces an impairment. In previous studies, the PROG/chow feeding choice task has been used to assess the ability of drugs to enhance selection of high effort PROG lever pressing (e.g. Randall et al. 2012, 2015). This task is especially well suited for such an assessment because unlike the FR5/chow feeding choice task, baseline lever pressing rates are relatively low on the PROG component due to the increasing response requirement of the PROG schedule. Thus, as the ratio work requirement gets gradually higher, animals eventually shift over to chow consumption (Randall et al. 2012, 2014,

2015). Previous research has demonstrated that the NET inhibitors desipramine and atomoxetine, as well as the SERT inhibitor fluoxetine, all fail to increase PROG lever pressing in rats tested on the PROG/chow feeding choice procedure (Yohn et al. 2016d). In contrast, drugs that inhibit DAT, including bupropion, lisdexamfetamine, PRX-14040, MRZ-9547, and GBR 12909 all have been shown to increase selection of PROG responding on this task (Sommer et al. 2014; Randall et al. 2015; Yohn et al. 2016b,c,d). In the present studies, ***(S)***-CE-123 was shown to significantly increase PROG lever pressing and decrease chow intake in rats tested on the PROG/choice procedure (**Fig. 3.4**). Furthermore, the effective dose of ***(S)***-CE-123 in both the TBZ reversal and PROG/chow feeding choice studies, 24.0 mg/kg, significantly increased extracellular DA in nucleus accumbens core as measured by microdialysis (**Fig. 3.5**). The magnitude of the increases in PROG lever pressing and accumbens core DA shown in the present studies with ***(S)***-CE-123 are comparable to those reported previously for 20.0 mg/kg bupropion (Randall et al. 2015). It is not clear why extracellular DA did not increase in the nucleus accumbens shell after injections of ***(S)***-CE-123, however, DA did substantially increase in the core, and previous work has shown that the core subregion is a critical site at which effort-based choice is regulated (Sokolowski and Salamone 1998; Ghods-Sharifi and Floresco 2010; Farrar et al. 2010; Randall et al. 2012; Nunes et al. 2013).

Several lines of evidence indicate that DAT blockers can have pro-motivational effects in animal models (Nunes et al. 2013; Randall et al. 2015; Sommer et al. 2014; Yohn et al. 2016a,b,c,d). Although DAT inhibition is a commonly used descriptor for a broad class of drugs, there is considerable heterogeneity within this group of compounds. For example, amphetamines such as d-amphetamine and methamphetamine are not only competitive inhibitors of DAT; they also are substrates that are transported into the neuronal terminal and stimulate release (Ferris et

al. 2011). Cocaine is a classical DAT inhibitor with a rapid onset and offset of action (Tanda et al. 2013). Unfortunately, most classical DAT inhibitors and releasing agents have a well characterized abuse liability (Todtenkopf and Carlezon 2006; Ostlund et al. 2014; Dong et al. 2017), which limits their therapeutic utility for treating motivational dysfunction in psychiatry. However, not all drugs that bind to the DAT share cocaine's behavioral profile or have a high potential for abuse. Over the last several years, atypical DAT inhibitors have been under development, which have characteristics that differ from cocaine. GBR12909 was developed as a potential treatment for cocaine addiction (Rothman et al. 2008). GBR12909 blocks many of the effects of cocaine, and on its own has produced mixed effects in terms of cocaine-like actions; it did not produce psychostimulant effects in people in the dose ranges tested (Sogaard et al. 1990; Preti 2000), and although it supported self-administration in primates, its efficacy was lower than that of cocaine (Woolverton et al. 2001). Although GBR12909 was discontinued as a potential treatment for cocaine abuse due to its cardiac effects, analogs of GBR12909 have been studied for their atypical binding characteristics (Rothman et al. 2008). The DAT has multiple functional conformations, and several benztropine analogs have been shown to bind to the DAT in a manner that is distinct from that of cocaine (Schmidt et al. 2008; Kohut et al. 2014). Some of these benztropine analogs have been shown to increase extracellular DA in nucleus accumbens, albeit over a much longer time course than cocaine (Tanda et al. 2013). Unlike cocaine, these drugs failed to induce conditioned place preference (Tanda et al. 2013).

***(S)***-CE-123 is an analog of modafinil, which also binds to the DAT with an atypical profile (Schmitt and Reith 2011; Cao et al. 2016). Modafinil inhibits DAT and increases extracellular DA over a broad time course (Mereu et al. 2017), and while this drug has been shown to have cognitive enhancing and pro-motivational effects, it has a relatively low abuse

liability (Müller et al. 2013; Mereu et al. 2013). Modafinil has been reported to improve fatigue symptoms in depressed patients (Lam et al. 2007), and recent studies from our laboratory have shown that modafinil can reverse the low-effort bias induced by TBZ in rats (Yohn et al. 2016c; Salamone et al. 2016a). When bound to the DAT, ***(S)***-CE-123 also has an atypical pattern compared to cocaine, in that it interacts with the negatively charged ASP79 locus (Kristofova et al., 2018). Moreover, ***(S)***-CE-123 acts as a highly selective atypical inhibitor of DAT relative to NET and SERT, and is more selective for DAT than modafinil (Kristofova et al. 2018). ***(S)***-CE-123 has been shown to enhance cognitive flexibility, improve memory acquisition and retrieval, and reduce impulsivity in rats (Nikiforuk et al. 2017; Kristofova et al. 2018), and in the present studies, it reverses the motivational impairments induced by TBZ and increases selection of high-effort PROG lever pressing.

In the present studies, a partial reversal of the effort-related motivational dysfunction induced by TBZ was achieved with ***(S)***-CE-123 administration in a dose range of 6.0-24.0 mg/kg. The efficacy of ***(S)***-CE-123 at restoring lever pressing appears to be lower than that of bupropion, GBR12909, lisdexamfetamine, methylphenidate and modafinil in rats tested on similar procedures (Nunes et al. 2013; Yohn et al. 2016a,b,c; Salamone et al. 2016a), although most drugs that block DAT tend to produce only partial reversals of the effects of TBZ (i.e., approximately 60-85% restoration of responding). Nevertheless, ***(S)***-CE-123 was highly efficacious at reversing the TBZ-induced increase in chow intake (**Fig. 3.2B**), which may indicate that this drug also has appetite suppressant actions. These potential appetite suppressant effects of ***(S)***-CE-123 would suggest that the increases in lever pressing seen in the FR5 and PROG/chow feeding choice studies are not due to an increase in appetite. Drugs that inhibit DAT bind across a broad range of affinities, which is potentially related to differences in potency seen

in behavioral experiments. High affinity drugs such as d-amphetamine, methylphenidate, PRX-14040, and GBR12909 have a relatively high potency for reversing the effects of TBZ (Yohn et al. 2016a,b,c,d; Salamone et al. 2016a). In contrast, compounds with lower DAT binding affinities such as bupropion, modafinil and ***(S)***-CE-123 tend to be less potent at reversing the effects of TBZ (i.e., they require higher doses; Nunes et al. 2013; Salamone et al. 2017a; present studies).

Future studies should examine a larger group of atypical DAT blockers to determine the overall relation between the neurochemical characteristics of these compounds (e.g. DAT affinity, selectivity and binding locus, effects on DAT trafficking (Mash et al., 2002; Vaughan et al., 2013), dynamics of effects on extracellular DA) and their effort-related behavioral effects. Furthermore, behavioral studies should be extended to include tasks that provide information about other behavioral effects, including locomotor activity, and most importantly, potential abuse liability. Drugs such as amphetamine and methylphenidate can increase selection of high-effort activity in humans (Wardle et al. 2011), and can improve motivational function in depressed patients (Stotz et al. 1999), but they also are well known for their abuse liability. In contrast, the atypical DAT blocker modafinil has been reported to improve motivational function in depressed patients (Lam et al. 2007), albeit without a strong abuse liability. The molecular interactions that occur as a result of the particular functional configuration of the DAT as it binds to drugs that block its action may be related to the diversity of patterns of abuse potential seen across multiple drugs (Tanda et al. 2013). Thus, the binding characteristics of drugs like modafinil and its analogs, such as ***(S)***-CE-123, suggest that novel compounds may ultimately be identified that are suitable for treating motivational dysfunction, and future studies should determine if ***(S)***-CE-123 shows signs have having substantial abuse potential.

## 8.4 Chapter 4 Discussion:

DAT inhibitors with atypical binding characteristics are being studied for their ability to ameliorate effort-related symptoms of fatigue and motivational dysfunction in rodents. The present work focused on the pharmacodynamic, neurochemical, and behavioral profile of the recently synthesized modafinil analog, (*S, S*)-CE-158. To determine the selectivity of (*S, S*)-CE-158 for the DAT, *in vitro* binding and monoamine transporters reuptake inhibition experiments were conducted using transfected HEK293 cells expressing human isoforms of DAT, NET, and SERT. In the present study, the $IC_{50}$ of (*S, S*)-CE-158 binding to the DAT was reported to be 0.052 µM, while its DAT inhibition was greater than 50-fold selective relative to NET (SERT reuptake inhibition was undetected; **Fig. 4.2, 4.3**). These patterns are consistent with results from inhibition assays measuring the $EC_{50}$ for inhibition of monoamine transporters of the previously synthesized compound, *(S)*-CE-123, which revealed a 30-fold selective inhibition of DAT relative to NET and more than 400-fold selective compared to SERT (Nikiforuk et al. 2017). By direct comparison, *(S, S)*-CE-158 is even more selective for the DAT than *(S)*-CE-123, supporting its potential use as a DAT inhibitor with limited off-target effects.

The FR5/chow feeding choice experiment was performed to investigate the effects of (*S, S*)-CE-158 for its ability to reverse the effort-related effects of TBZ. Consistent with previous findings (Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2015a,b,c; Rotolo et al. 2019), administration of 1.0 mg/kg TBZ shifted choice behavior, decreasing high effort lever pressing and increasing low-effort chow intake (**Fig. 4.4**). Previous research has shown that the effects of TBZ on effort-related choice are not due to actions on the intake of or preference for the two foods used in the FR5/choice task (Nunes et al. 2013), discrimination of reinforcer magnitude or reference memory (Yohn et al. 2015a), or sucrose preference or hedonic reactivity (Pardo et al.

2015). Furthermore, the effects of TBZ on effort-based tasks do not resemble the effects of reinforcer devaluation by reduction in food motivation, or appetite suppressant drugs (Randall et al. 2012, 2014; Yang et al. 2020). For these reasons, and also because TBZ induces motivational symptoms such as fatigue in humans (Chitnis and Karunapuzha, 2009; Frank, 2010; Chen et al. 2012), TBZ was used in the present experiment to provide a challenge to the tendency of rats to exert physical effort when given a choice.

Co-administration of 8.0 mg/kg (*S*, *S*)-CE-158 with TBZ resulted in a significant reversal of the effects of TBZ on both lever pressing and chow intake. The magnitude of this effect can be observed by comparing the average number of lever presses at the dose that produced a significant reversal to the number of lever presses in the vehicle group. Here, the co-administration of 8.0 mg/kg (*S*, *S*)-CE-158 with TBZ yielded a lever pressing average which was 85% of the vehicle/vehicle average (**Fig. 4.4a**), whereas Rotolo et al. (2019) reported only a 40% restoration of the vehicle/vehicle lever pressing average when the highest dose of *(S)*-CE-123 (24.0 mg/kg) was co-administered with TBZ. The ability of *(S)*-CE-123 to only partially reverse the effects of TBZ was attributed to possible appetite suppressant actions, which were not observed in the present experiment. Moreover, the efficacy of (*S*, *S*)-CE-158 for reversing the effort-related motivational dysfunction induced by TBZ is comparable to a group of DAT inhibitors including GBR12909, lisdexamfetamine, and methylphenidate in terms of their relatively large effects in terms of reversing the effects of TBZ (Salamone et al., 2016a; Yohn et al., 2016a,b,c).

To measure its effect when administered alone, (*S*, *S*)-CE-158 was administered to rats trained on the concurrent PROG/chow feeding choice task. This task is well suited for assessing the effects of a drug when administered alone, in terms of increasing the motivation to exert high

levels of effort on a more highly demanding task. In the present study, (*S*, *S*)-CE-158 significantly increased the selection of the high-effort option (lever pressing) and a reduced selection of the low-effort option (chow intake) at both the 4.0 mg/kg and 8.0 mg/kg doses (**Fig. 4.5**). These results are consistent with previous experiments demonstrating that DAT inhibitors such as bupropion, lisdexamfetamine, MRZ-9547, and PRX-14040 significantly increased PROG lever pressing and decreasing chow consumption (Sommer et al. 2014; Randall et al., 2015; Yohn et al., 2016b,c,d), whereas drugs that inhibit NET and SERT did not (Yohn et al. 2016d). These results also are consistent with the findings of Cagniard et al. (2006), who reported that DAT knockdown increased selection of lever pressing in mice tested on an effort-based choice task, and with Wardle et al. (2011), who reported that amphetamine increased selection of high-effort activity in human participants. Furthermore, it was shown that systemic administration of (*S*, *S*)-CE-158 at a dose of 8.0 mg/kg significantly increased extracellular DA in the nucleus accumbens core as measured by microdialysis (**Fig. 4.6**). The peak increase in DA at 30-90 minutes post-injection is aligned with the time course used for the behavioral pharmacology experiments (i.e. 30 min injection lead time prior to a 30 min task). Systemic administration of DAT inhibitors in rodents has been reported to increase extracellular DA in the nucleus accumbens core, which has been associated with increased motivation (Yohn et al. 2016d; Rotolo et al. 2019) and pro-cognitive effects such as enhanced recognition memory (Carnats-Perna et al. 2019).

The ability of *(S)*-CE-123 and (*S*, *S*)-CE-158 to increase motivation and ameliorate effort-related impairments can be seen as complimentary to the known effects of their parent compound, modafinil. In addition to its prescribed indication as a treatment for narcolepsy, modafinil is frequently used by many individuals as a nootropic, for off-label effects such as

cognitive enhancement (Sahakian and Morein-Zamir, 2011; Battleday and Brem, 2015; Sousa and Dinis-Oliveira, 2020; Teodorini et al. 2020). Memory and cognitive flexibility have been described as effects of both the *S*-configuration and the racemic mixture of the thiazole-containing modafinil analog, CE-123 (Nikiforuk et al. 2017; Kristofova et al. 2018; Carnats-Perna et al. 2019). Interestingly, it has been suggested that modafinil's effects related to cognitive processes and task engagement may be dependent on fronto-striatal circuitry, rather than hippocampal (Müller et al. 2013), which further supports the utility of these drugs as treatments for effort-related dysfunction. Moreover, it has been suggested that modafinil and its derivatives possess anti-inflammatory properties *in vitro* (Jung et al. 2012) and *in vivo* (Zager et al. 2017; Han et al. 2018; Brandão et al. 2019), and may reduce multiple sclerosis fatigue in humans (Shangyan et al. 2018), underlying possible utility of these compounds to attenuate symptoms of various disorders that are propagated by inflammatory cascades. Therefore, results from the current studies may be useful to guide future assessments of atypical DAT inhibitors to expand the clinical indications for modafinil and similar compounds.

In summary, these experiments contributed to the characterization of a recently developed group of atypical DAT inhibitor compounds, by demonstrating the ability of (*S*, *S*)-CE-158 to reverse the effort-related effects of TBZ, and increase high-effort responding in tasks measuring effort-based choice. Additionally, it was shown that systemic administration of (*S*, *S*)-CE-158 increased extracellular DA in the nucleus accumbens during the behaviorally active time course. The behavioral assessment of atypical DAT inhibitors such as analogs of benztropine, GBR12909 and modafinil has led to the discovery that not all inhibitors of DA uptake show signs of abuse liability to the same extent as the classical DAT inhibitors that are major psychostimulants such as cocaine (Sogaard et al. 1990; Preti 2000; Woolverton et al. 2001;

Loland et al. 2008; Schmitt et al. 2008; Esumi et al. 2012; Tanda et al. 2013; Mereu et al. 2013). These results suggest it is possible that atypical DAT inhibitors may be effective at reducing motivational impairments with minimal induction of major psychomotor side effects or abuse liability. Ultimately, this research will guide future studies to advance target identification for treatments of motivational dysfunction. These and other similar findings could lead to the development of atypical DA uptake blockers that could potentially treat motivational symptoms of depression, chronic fatigue syndrome, or other disorders, while reducing undesirable side effects.

**8.5 Chapter 5 Discussion:**

Motivational dysfunctions such as anergia, fatigue, and low-effort bias are debilitating treatment-resistant psychiatric symptoms that span multiple disorders, including MDD, PD, schizophrenia and MS (Treadway et al. 2012; Yang et al. 2014; Zimmerman et al. 2015; Corfield et al. 2016; Chong et al. 2016; Barch et al. 2017; Le Heron et al. 2019). In view of data from animal models and human studies indicating that DA systems regulate behavioral activation and effort-related function, DAT inhibitors considerable potential as treatment targets. The present studies showed that the novel compound CT-005404 selectively binds to DAT relative to NET and SERT (**Table 5.1**), and also increases extracellular DA with a prolonged time course as measured by microdialysis (**Fig. 5.1**). Furthermore, CT-005404 reversed the low-effort bias induced by the VMAT-2 inhibitor TBZ (**Fig. 5.2**), as well as the suppression of lever pressing induced by the pro-inflammatory cytokine IL-1β (**Fig. 5.3**). When administered alone, CT-005404 increased the tendency to select high-effort PROG lever pressing (**Fig. 5.4**). Taken together, these results suggest that CT-005404 has the profile of a drug that could be useful for the treatment of effort-related motivational dysfunctions in humans.

Consistent with previous studies (Nunes et al. 2013), 1.0 mg/kg TBZ shifted effort-based choice, significantly decreasing FR5 lever pressing while increasing intake of the concurrently available chow. The ability of TBZ to induce this effect is not dependent on changes in food intake or food preference, and does not resemble the effects of appetite suppressant drugs or reinforcer devaluation on this task (Salamone et al. 1991, 2002; Sink et al. 2008; Nunes et al. 2013). Co-administration of CT-005404 with TBZ led to a significant but partial reversal of the effects of TBZ. Compared to TBZ alone, rats administered TBZ plus 15.0 and 30.0 mg/kg doses of CT-005404 showed significant increases in lever pressing and decreases in chow intake (**Fig. 5.2**). Consistent with the gradual and prolonged elevations in extracellular DA that were seen in the microdialysis experiment, CT-005404 was able to reverse the effects of TBZ over the period of 3-4.5 hours after its p.o. administration. The ability of CT-005404 to show positive effects in the TBZ model is consistent with previously published studies showing that a variety of compounds that block DAT, including the catecholamine uptake inhibitor bupropion (Nunes et al. 2013; Yohn et al. 2016b), and the DAT inhibitors lisdexamfetamine, GBR 12909, and PRX-14040 (Yohn et al. 2016a,b,c,d), all act to reverse the effort-related effects of TBZ.

Pro-inflammatory cytokines play a significant role in mediating central and peripheral immune responses, but it has become evident that these proteins also are involved in the manifestation of psychopathological symptoms including anergia, fatigue, and amotivation (Dantzer 2001; Dantzer et al. 2009; Felger and Miller 2012; Felger and Treadway 2017). Increased expression of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interferon alpha (IFN-α), and IL-1β in patients with major depression (Yang et al. 2007; Dantzer et al. 2008; Dowlati et al. 2010; Cattaneo et al. 2013; Rossi et al. 2017). mRNA levels of inflammatory markers including IL-1β, macrophage inhibiting factor,

and TNF-α, were reported to be negatively correlated with antidepressant treatment response (Cattaneo et al. 2013). TNF-α, IL-6, and IL-1β are also elevated in patients with MS, and increased levels of TNF-α and IL-1β are correlated with clinical depression symptom severity in MS patients (Rossi et al. 2017). IL-1β was shown to decrease lever pressing and slightly increase chow intake in rats tested on the FR5/chow feeding choice task (Nunes et al. 2014). In the present studies, IL-1β was used to provide an inflammatory challenge, which may be relevant for understanding disorders associated with elevated inflammation including MDD and MS. CT-005404 was co-administered with IL-1β (4.0 µg/kg) at doses ranging from 7.5-30.0 mg/kg i.p. IL-1β administration significantly decreased lever pressing, without altering chow intake. The 15.0 and 30 mg/kg doses of CT-005404 partially but significantly reversed the effects of IL-1β, significantly increasing lever pressing compared to treatment with IL-1β alone (**Fig. 5.3**). Evidence indicates that brain DA transmission interacts with the effects of pro-inflammatory cytokines. Pro-inflammatory cytokine signaling has a modulatory effect on some aspect of monoamine synthesis and/or transmission (Rossi et al. 2010; Gentile et al. 2015; Felger and Treadway 2017). Extracellular levels of DA in nucleus accumbens were reduced by administration of IL-6 in rats at a dose that suppressed lever pressing, and the DAT inhibitor methylphenidate reversed the lever pressing suppression induced by IL-6 (Yohn et al. 2016), which is consistent with the ability of CT-005404 to reverse the suppression of lever pressing induced by IL-1β.

The PROG/chow feeding choice task provides a stronger effort-related challenge than the FR5/chow choice task, because the lever pressing work requirement gradually increments throughout the session. This task generates substantial variability in PROG lever pressing output (Randall et al. 2012, 2014, 2015), which also was observed in the present results. Administration

of CT-005404 alone led to a significant linear dose-related increase in PROG lever pressing, as well as a decrease in chow intake. The highest dose of CT-005404 significantly increased PROG lever pressing and decrease chow intake relative to vehicle (**Fig. 5.4**), suggesting that the administration of this novel DAT inhibitor increased the tendency of animals to select the high-effort lever pressing component of the task. This finding is consistent with previous studies showing that selection of PROG lever pressing relative to chow intake was increased by several drugs that inhibit DAT, including bupropion, lisdexamfetamine, GBR 12909, and PRX-14040 (Randall et al. 2015; Yohn et al. 2016b,c,d). Taken together, these studies demonstrate that DA transmission exerts a bi-directional control over effort-based decision making, i.e., that interference with DA transmission induces a low-effort bias, while augmentation of DA transmission by blockade of DAT increases selection of high-effort lever pressing. These findings also are consistent with Wardle et al. (2011), who reported that amphetamine increased selection of high-effort activity in human participants.

In summary, CT-005404 is a novel DAT inhibitor that binds selectively to DAT compared to NET and SERT, increases extracellular DA in nucleus accumbens, reverses the suppression of lever pressing induced by TBZ and IL-1β, and when administered alone, increases the tendency to select high-effort PROG lever pressing. Thus, CT-005404 has a profile similar to that shown by bupropion, modafinil, and methylphenidate, which are drugs that have been shown to improve motivational function in humans. Future research should determine if CT-005404 shows signs of abuse liability. It is possible that CT-5404 or other novel DAT inhibitors could ultimately be useful as treatments for motivational dysfunction in humans suffering from MDD, Parkinsonism, MS, or other disorders.

**8.6 Chapter 6 Discussion:**

This experiment was undertaken to develop a modified PROG task to be used for assessments of effort-based decision making in rats, and to assess the effects of DA depletions on several measures of high-effort responding during the PROG session. Although most studies with PROG schedules are relatively open ended in terms of session times, this specific task was developed in order to obtain break points from animals during a 30-minute session, so that in the future recording experiments can readily be done during the period of time in which rats show break points. Although it has sometimes been thought that PROG break points are measures of "reward" or "reinforcement efficacy", PROG break points are most directly a measure of how much work an organism will do to obtain reinforcement (Stewart 1974; Salamone 2006). For example, increasing the work requirement by increasing the height of the lever has been reported to decrease progressive ratio break points (Skjoldager, Pierre and Mittlman 1993; Schmelzeis and Mittleman 1996). Thus, PROG tasks are useful in studying the activational aspects of motivation and the tendency to work for food reinforcement (Salamone 2006; Salamone and Correa 2002; Salamone et al. 2016a), and how those the tendency to work for reinforcement is sensitive to various pharmacological manipulations. Though the PROG/chow feeding choice task has been used as a reliable model of effort-related choice behavior in animals for a number of years (Randall et al. 2012, 2014, 2015; Sommer et al. 2014; Yohn et al. 2016b,c,d, 2018), this study was the first to examine the effects of altering ratio requirements on PROG work output (e.g. lever pressing, time of last response, and breakpoint ratio) through distinct training phases in the same cohort of rats over a number of weeks, and the first to examine the effects of TBZ on high-effort work output on this modified schedule.

By separating the training into separate phases, we were able to determine the point at which the parameter modifications were difficult enough to cause the majority of rats to cease responding, or 'break' during a 30-minute session. As depicted in **Figure 6.1**, the PROG (N=1; I=4) schedule was chosen as the schedule to be used for the behavioral pharmacology experiments. A four-day sample of baseline data taken from the 'N=1; I=4' training phase indicated that only three of sixteen rats did not break prior to the end of the 30-minute session. It was also observed that the rats that demonstrated breakpoints prior to the session end typically ceased responding within the first three quarters of the session. These data should be used to inform future studies of effort-related choice, ultimately allowing for direct observations of the behavioral and electrophysiological changes that are taking place as an animal shifts away from engaging in a high-effort task.

The behavioral pharmacology experiment assessed the effects of tetrabenazine administration on total lever presses, time of last response, and break point ratio. Tetrabenazine administration resulted in significant reductions of all three measures (**Fig. 6.2**). These findings indicate that reduced DA transmission causes animals to exert less effort to overcome an instrumental response cost, effectively modeling the motivational deficits observed in individuals with neurological or psychiatric disorders. While treatment with DA antagonists or depleting agents causes animals to shift from choosing high- to low-effort options in tasks motivated by food, previous research has shown that this is not due to reduced appetite or alterations in preference or hedonic taste reactivity as the animals' preference for the high-density arm or high carbohydrate pellet is still intact (Salamone et al. 1994; Berridge and Robinson, 1998; Randall et al. 2012, 2014; Nunes et al. 2013; Pardo et al. 2015; Yohn et al. 2015a). Similarly, it is important to note that humans with depression still maintain preference and the ability to perceive hedonic

stimuli, but lack the drive to exert the effort required to obtain it (Treadway and Zald, 2011; Treadway, 2012). When administered a drug that blocked catecholamine synthesis, human subjects were still able to detect the hedonic properties of cigarette smoking, and still experienced feelings of craving, but demonstrated lower response break points on the PROG schedule, indicating a reduced willingness to work for cigarettes (Venugopalan et al. 2011). In summary, DA depletions cause reductions in rats' willingness to exert effort on the PROG lever pressing procedure, which can be compared to human studies of psychopathology related to the exertion of physical effort. In this regard, it is interesting to note that clinical studies have shown that willingness to work for monetary reward on a PROG task was impaired in people with depression and bipolar depression (Hershenberg et al. 2016).

The development of behavioral tasks that requires high levels of effort is critical for inducing a low-effort bias in animals. The systematic adjustments that were made to the PROG reinforcement schedule in this study have provided useful information about the effects of increasing: a) the number of reinforcers delivered at each ratio requirement (N); and b) the increment at which the ratio requirement increases after N reinforcers are delivered (I). In addition, the modified PROG task developed here will foster the study of EEG activity in behaving rats as they shift from high-effort lever pressing to the low-effort alternative, which will serve as a pre-clinical model of the physiological and neural changes that are relevant in human fatigue, anergia, and psychomotor slowing. Ultimately, these studies may allow for the assessment of identifiable patterns of prefrontal/frontal activity that may be relatable to the exertion of effort.

## 8.7 Chapter 7 Discussion:

Our initial studies involving EEG recording have focused on conducting power spectral density analyses of signals obtained from frontal and parietal cortex, and determining the effects of TBZ, in untrained freely moving animals. These experiments were intended to be preliminary studies that allow for the development of EEG and data analysis methods that could lay the groundwork for future studies performed on trained animals performing on effort-related choice tasks. Ultimately, this research could allow for the development of EEG markers of effort-based choice that are translatable to humans. Furthermore, future research will study the behavioral and EEG effects of pharmacological manipulations that affect effort-based choice in trained animals, so that we can create larger, more robust variations in approach motivation that will potentially allow us to identify more sensitive electrophysiological biomarkers.

Results from the **Chapter 7** studies identified at least one major difference in relative frequency band power as a result of TBZ administration, and also focused on inter-hemispheric coherence based on electrode placements. Data from six rats treated with 1.0 mg/kg TBZ demonstrated a reduction of power in a range of 7-8 Hz relative to baseline (**Fig. 7.1**). Although 8 Hz frequency bands can be categorized as within the alpha frequency range, theta oscillations have been reported within a frequency range of 4-12 Hz (Buzsaki et al. 1983; Penley et al. 2011). Theta and gamma oscillations originating from hippocampal structures have been implicated in activities such as locomotion and task engagement in the awake rat (Penley et al. 2011; Hinman et al. 2013; Jacobson et al. 2013; Schmidt et al. 2013), and thus should be considered as possible contributors to the cortical EEG signals observed in the present studies. Furthermore, our results suggested that TBZ did not seem to affect inter-hemispheric coherence of EEG signal from skull screw electrodes. Pharmacological manipulations and lesion studies have been shown to affect

relative beta, theta, and gamma frequency power as well as measures of coherence as measured by EEG and LFP signals in the rat brain (Pálenícek et al. 2011, 2013; Hinman et al. 2013; West et al. 2018). For example, the DAT inhibitor amphetamine caused an increase in theta and alpha band coherence and a decrease in beta coherence, which was linked to an increase in locomotion and extracellular accumbens DA (Pálenícek et al. 2013). These studies emphasize the importance of studying both intra- and inter-hemispheric coherence in future studies assessing the effects of TBZ in behaving rats. Nevertheless, the data shown in **Figure 7.2** suggest that the position of electrodes greatly affected the signal coherence, as demonstrated by the strongest signal coherence between two frontal electrodes positioned on either side of the midline, and two parietal electrodes positioned on either side of the midline, regardless of drug treatment. Results from this initial study demonstrate the feasibility of doing recording experiments with a relatively high degree of spatial resolution (i.e. differentiating prefrontal and parietal cortical areas) and a notable sensitivity to changes induced by pharmacological manipulations.

Considering that the data reported here reflect EEG activity in an awake rat under baseline conditions, it is expected that rats behaving on the PROG task will show marked differences in EEG activity across multiple frequency bands. For example, animals engaging in high-effort PROG lever pressing may demonstrate lower frontal-frontal coherence, as human studies have posited a relation between frontal alpha asymmetry and motivated behaviors, as well as a reduced propensity to exhibit depressive symptoms (Pizzagalli et al. 2005; Nelson et al. 2018; Gheza et al. 2019). Furthermore, changes to beta power may be observed in animals engaging in the PROG task under baseline (non-drug) conditions and after treatment with TBZ. Beta oscillations in cortical-basal ganglia circuits have been highlighted as a critical modulator of movement-related activity (Leventhal et al. 2012; Schmidt and Berke et al. 2017), and

abnormal beta rhythms have been linked to the motor impairments seen in Parkinson's disease (Levy et al. 200l; West et al. 2018) and to pathological motor output in animals during task engagement (Leventhal et al. 2012). Therefore, the reduction of DA transmission in striatal areas caused by TBZ may result in alterations in beta power in rats that are correlates of a low-effort bias during PROG performance. Although TBZ primarily affects subcortical DA transmission in DA-rich striatal areas (Pettibone et al. 1984; Tanra et al. 1995), a sparse DA innervation also is present in areas of cortex other than prefrontal regions, with an anterior/posterior gradient (Vitrac and Benoit-Marand 2017), and thus TBZ may affect cortical DA transmission in ways that have not yet been elucidated.

The initial step in this study was to establish reliable procedures for recording frontal cortex EEG signals in awake rats. Now that initial procedures have been established, future work will aim to record EEG activity while rats are performing on a high-effort lever pressing task (PROG) under baseline conditions and after pharmacological challenges that produce motivational deficits. Since PROG responding is characterized by initial periods of responding followed by a cessation of responding (i.e., "breaking" or ratio strain), a major goal of this future work will be to determine if there are differences in frontal cortical EEG activity during the initial period of responding vs. the period during and after the breakpoint. Ideally, this experiment will involve cohorts of male and female rats that will be trained on the modified PROG task developed in the studies outlined in **Chapter 6**. Once sufficiently trained, rats will be surgically implanted with up to six cortical EEG electrodes adjacent to the frontal cortex and parietal cortex as additional reference points. After recovery, rats will be tested on their respective behavioral tasks under baseline (non-drug) conditions and EEG activity will be recorded throughout the behavioral sessions. Then, rats will be tested on their respective

behavioral tasks under pharmacological challenge with the VMAT-2 inhibitor TBZ. Additional studies will involve administration of TBZ to induce lower breakpoints, in order to determine if the changes in PROG responding induced by TBZ covary with changes in EEG activity. Based upon the Pizzagalli et al. (2005), Nelson et al. (2018), and Gheza et al. (2019) papers, as well as the present studies, it is hypothesized that engagement in high-effort lever pressing activity in the PROG will be marked by changes in frontal EEG activity, and possibly changes in parietal cortex, and in frequency bands other than alpha, such as theta beta, and gamma, as well. Furthermore, it is hypothesized that when animals trained on the PROG task reach their break point ratio, stop lever pressing, and shift to chow consumption, there will be changes in frontal EEG activity. Lastly, it is hypothesized that TBZ will shift effort-related choice behavior, decreasing lever pressing and increasing chow intake, which will thus alter the pattern of frontal cortex EEG activity relative to baseline conditions. These results could lead to a greater understanding of the circuit mechanisms that underlie effort-based aspects of motivation, while providing preclinical animal data leading to the development of physiological biomarkers of exertion of effort that can be easily translatable to human clinical research.

### 8.8 General Discussion and Conclusions

Effort-related motivational dysfunctions such as anergia, lassitude and fatigue are hallmark symptoms of depression, parkinsonism, schizophrenia, multiple sclerosis, and other disorders. Unfortunately, the most commonly prescribed medications do not effectively treat these symptoms, and can sometimes exacerbate them (Fava et al. 2014). Preclinical models of effort-based decision making can be used to study the amotivation produced by some of the conditions associated with depression. The use of behavioral tasks to model specific facets of

depression and other psychiatric disorders is aligned with the NIMH Research Domain Criteria (RDoC) initiative, which emphasizes the importance of characterizing the neural processes that underlie the development of specific psychiatric symptoms. Importantly, tasks that have been designed to measure effort-related decision making are important in assessing the therapeutic potential of novel pharmacological compounds for their ability to mitigate effort-related deficits in animals.

Considerable evidence supports the role of DA in modulating motivational processes. Drugs that reduce central DA transmission demonstrate a tendency to disturb motivational functions in animals (Nunes et al. 2013; Randall et al. 2012, 2014; Yohn et al. 2015a,b) and in humans (Artaloytia et al. 2006; Frank 2009, 2010; Guay, 2010; Chen et al. 2012). Consistent with previous literature, the DA $D_2$ antagonist haloperidol reduced high-effort responding on the FR5 feeding task, and shifted rats' behavior from the high effort (lever pressing) to low effort (chow intake) alternative on the concurrent FR5/chow feeding choice task (Chapter 2). In several experiments, the VMAT-2 inhibitor TBZ was shown to alter effort-based choice on the FR5/chow feeding choice task (Chapters 3-5) and on a PROG lever pressing schedule (Chapter 6). Reductions in lever pressing also were induced by administration of the pro-inflammatory cytokine IL-1β (Chapter 5). These findings have been validated by clinical studies of effort-related decision making showing that depressed individuals demonstrate a reduced likelihood of selecting high effort alternatives (Treadway et al. 2012a; Yang et al. 2014).

Numerous studies have shown that administration of classical DAT inhibitors are able to reverse the effects of TBZ (Nunes et al. 2013, Yohn et al. 2016a,b; Salamone et al. 2016b) and of pro-inflammatory cytokines (Yohn et al. 2016c), but these drugs tend to produce undesirable side effects. Therefore, a novel class of DAT inhibitor compounds was investigated in Chapters 3-5.

The novel modafinil analogs *(S)*-CE-123 and *(S, S)*-CE-158 were able to reverse the motivational deficits induced by TBZ on the FR5/chow feeding choice task, increase PROG responding, and increase extracellular accumbens DA as measured by microdialysis (Chapter 3-4). In addition, the recently synthesized atypical DAT inhibitor CT-005404 reversed a suppression of lever presses produced by IL-1β, while also demonstrating the ability to reverse the effort-related effects of TBZ, increase PROG responding, and increase extracellular DA in nucleus accumbens (Chapter 5). Together, the findings from Chapters 3-5 serve as important preclinical evidence for atypical DAT inhibitor compounds in support of their potential utility as treatments for symptoms of fatigue and amotivation seen in depression, schizophrenia, multiple sclerosis, and other disorders. Future studies need to focus on the potential abuse liability of these compounds.

As discussed above, pharmacological conditions such as the administration of DA depleting agents or antagonists, or pro-inflammatory cytokines, can induce a low-effort bias in rats (Salamone et al. 2016a,b,c; Yohn et al. 2016a,b,c), and these effects can be reversed by co-administration of several drugs that facilitate DA transmission (Chapters 3-5). Despite a growing body of animal work demonstrating that the exertion of effort and selection high-effort options is dependent upon forebrain circuits that involve mesolimbic DA inputs to nucleus accumbens, ventral pallidum, amygdala, and prefrontal cortex (Salamone et al. 1991, 1994; 2007, 2016a,b, 2018; Walton et al. 2003; Floresco and Ghods-Sharifi 2007; Mingote et al. 2008; Farrar et al. 2008, 2010; Winstanley and Floresco 2016), potential physiological markers of low effort bias that can be readily translatable to human clinical research have not been elucidated. In light of this, Chapters 6-7 experiments employed a novel operant lever pressing task and EEG methods to aid in the development of physiological markers associated with altered DA transmission and effort-related dysfunction. In Chapter 6, a PROG task was developed that would require very

high levels of baseline lever pressing, causing rats to exhibit a breakpoint during a 30-minute session. TBZ shifted rats away from high-effort responding, reducing total number of lever presses, time of last response, and breakpoint on the modified PROG schedule (Chapter 6). The modified PROG lever pressing schedule developed in these experiments will be implemented in future studies of electrophysiological recordings in awake, behaving rats. In Chapter 7 experiments, cortical EEG was measured in rats under baseline and DA depletion conditions (i.e., administration of TBZ). Our findings suggested that there are several notable differences in the cortical EEG markers associated with altered DA transmission in rats, which may ultimately contribute to our understanding of how DA transmission in the ventral striatum and striatopallidal circuits modulate cortical functions involved in motivational dysfunctions in humans.

Finally, the aim of Appendix Experiments 1-3 was to investigate the effects of several non-dopaminergic drugs on effort-related choice behavior in rodents. Appendix Experiments 1 demonstrated that the SERT inhibitor fluoxetine suppressed FR5/chow lever pressing in male and female rats. Furthermore, Appendix Experiments 1 and 2 demonstrated that the effort-related effects induced by drugs such as fluoxetine, haloperidol, tetrabenazine, and risperidone could not be reversed by reversed by 5-$HT_{2A}$ or 5-$HT_{2C}$ antagonists, or the 5-$HT_{2A}$ inverse agonist pimavanserin. These findings have been corroborated by recent studies in our lab that have also shown the negative effects of serotonergic drugs on running wheel choice behavior. Lastly, the kappa opioid receptor antagonist BT was unable to reverse motivational impairments induced by tetrabenazine and risperidone on the concurrent FR5/chow feeding choice task. The present results suggest that there may be limited therapeutic utility for these particular modulators of

serotonin receptors or kappa opioid receptors in terms of their ability to reverse effort-related dysfunctions, further emphasizing the importance of DA in regulating effort-related functions.

In summary, the findings from the experiments described above highlight the critical role of DA in effort-related choice behaviors. DA transmission exerts a bi-directional modulatory control over exertion of physical effort in rats performing on instrumental tasks. Moreover, the behavioral paradigms used throughout this body of work have demonstrated their utility as preclinical tools for studying effort-related motivational impairments and identifying potential treatments. The conclusions that can be drawn from these experiments will aid in the development of novel therapeutic strategies for motivational impairments and will contribute to the growing body of literature on biomarkers related to fatigue and anergia observed in numerous psychiatric and neurological disorders that can be readily translated to human studies.

# Appendices

*Summary of Appendices*

Appendix Experiments 1-3 sought to assess the effects of several non-dopaminergic drugs on effort-related choice in rodents. **Appendix Exp 1** demonstrated that the administration of serotonin (5-HT) transport inhibitors (SERT inhibitors or SSRIs) reduced lever pressing and did not affect chow intake, but that this effect could not be reversed by the administration of selective serotonin receptor inhibitors (5-$HT_{2A}$ or 5-$HT_{2C}$). **Appendix Exp 2** examined the effort-related impairments induced by various pharmacological agents such as fluoxetine, haloperidol, risperidone, pilocarpine, and tetrabenazine, and showed that the 5-$HT_{2A}$ inverse agonist pimavanserin was unable to reverse the effort-related effects of any of these drugs. **Appendix Exp 3** examined kappa opioid receptor function in effort-related aspects of motivation by assessing a kappa opioid receptor antagonist for its ability to reverse the effects of tetrabenazine, or the effects of risperidone. Taken together, the negative drug reversal results from **Appendix Exp 1-3** emphasize the importance of DA in the regulation of effort-related behaviors, suggesting the need to focus on dopaminergic manipulations in developing treatments for the motivational dysfunction seen in depression and other disorders.

**Appendix 1: Investigation of SSRI-induced fatigue in effort-related choice tasks, and of several selective serotonin receptor antagonists as potential treatment strategies.**

## 1.1 Introduction

Major depressive disorder (MDD) often includes symptoms such as retardation, fatigue, lassitude, loss of energy and effort-related deficits, which commonly affect motivational and psychomotor functions (Stahl 2002; Demyttenaere et al. 2005; Salamone et al. 2006; Treadway and Zald 2011; Treadway et al. 2012a; Fava et al. 2014). The severity of such effort-related symptoms in depression is highly correlated with problems in social function, employment, and treatment outcomes (Tylee et al. 1999; Stahl 2002), and it has been observed that these debilitating symptoms are highly resistant to treatment (Stahl, 2002; Nutt et al. 2007; Fava et al. 2014). In fact, serotonin (5-HT) uptake inhibitors such as fluoxetine (FLX; Prozac) are relatively ineffective for treating motivational dysfunction, and can induce or exacerbate these symptoms (Daly et al. 2011; Padala et al. 2012; Stenman and Lilja 2013; Rothschild et al. 2014; Fava et al. 2014). Effort-related motivational symptoms are present in other disorders in addition to depression, including bipolar disorder, schizophrenia, Parkinsonism, chronic fatigue syndrome and multiple sclerosis (Salamone et al. 2006, 2010, 2016b; Friedman et al. 2007; Tellez et al. 2008; Green et al. 2015). For these reasons, it is critical to examine the facets of effort-related dysfunctions in animal models in order to improve upon the currently available treatment options for these disorders.

The neural basis of the effort-related dysfunctions in psychopathology are still being characterized, nevertheless, considerable preclinical and clinical evidence implicates central dopamine (DA), basal ganglia, and related corticolimbic circuits (Rogers et al. 1987; Rampello et al. 1991; Brown and Gershon 1993; Hickie et al. 1999; Caligiuri and Ellwanger et al. 2000; Volkow et al. 2001; Schmidt et al. 2001; Brody et al. 2001; Salamone et al. 2006, 2016b; Tellez et al. 2008; Treadway and Zald 2011; Chong et al. 2015). In addition to DA, other neurotransmitters such as glutamate, GABA, and 5-HT have also been implicated in the pathogenesis of MDD (Cowen and Browning 2015; Milak et al. 2016; Zheng et al. 2016; Belujon and Grace 2017; Pan et al. 2018), but their distinct roles in the regulation of motivational/psychomotor symptoms have not been fully elucidated. In view of the severe disease burden presented by depression (Wittchen et al. 2011; Marcus et al. 2012), the prevalence of motivational symptoms such as anergia and fatigue in depression and other disorders, and the relative resistance of these motivational/psychomotor symptoms to many common drug treatments, it seems evident that this line of work will ultimately improve the outlook for patients suffering from these debilitating symptoms.

Over the past several decades, a considerable body of work has focused on characterizing the role of various neurotransmitters including dopamine (DA), 5-HT, norepinephrine (NE), adenosine, and GABA, in the exertion of effort and effort-related decision making (Salamone and Correa 2002; Salamone et al. 1991, 2002, 2007, 2009a,b). Behavioral tasks have been developed in animals to study effort-related functions in animals, including operant procedures with different work requirements, and tests of effort-related decision making that allow animals to choose between high-effort alternatives that lead to more highly valued rewards vs. low-effort alternatives that lead to less valued rewards (i.e., less preferred or lower in magnitude). Tests

involving operant behavior and T-maze performance have demonstrated that low doses of DA antagonists, as well as DA depletions or antagonism in nucleus accumbens, reduce the exertion of effort and alter effort-related choice, biasing animals towards low-effort alternatives (Salamone and Correa 2002, 2012a; Salamone et al. 2007, 2009a,b, 2012). It has been suggested that tests of effort-based decision making in rodents could be useful as models of the effort-related motivational symptoms of depression (Salamone et al. 2003, 2006, 2007, 2012, 2015, 2016a,b,c; Salamone and Correa 2012). Reduced selection of high-effort activities in rodents is induced by manipulations associated with depression, including stress (Shafiei et al. 2012; Bryce and Floresco 2016), the pro-inflammatory cytokines IL-1β and IL-6 (Nunes et al. 2014; Yohn et al. 2016c), and the vesicular monoamine transport (VMAT-2) inhibitor tetrabenazine (Nunes et al. 2013b; Randall et al. 2014; Yohn et al. 2015a,b). These observations are validated by clinical data demonstrating that people with major depression show a reduced likelihood of selecting high effort alternatives when assessed in tests of effort-related decision making (Treadway et al. 2012a; Yang et al. 2014).

Recent studies from the Salamone laboratory have focused upon the effort-related effects of drugs that block the transporters for DA (DAT), NE (NET), and 5-HT (SERT). The effort-related effects of tetrabenazine can be reversed by co-administration of the antidepressant bupropion (Nunes et al. 2013b; Randall et al. 2014; Yohn et al. 2015a,c), which blocks DAT and NET, and also by the DAT blocker GBR12909 (Yohn et al. 2016a). However, the SERT inhibitors fluoxetine and escitalopram fail to reverse the effort-related effects of tetrabenazine, and instead tend to exacerbate them (Yohn et al. 2016a,b). Furthermore, evidence indicates that while DA exerts a bi-directional modulation over effort-related decision making, 5-HT does not. Increased selection of high-effort choices can be enhanced by augmenting DA transmission with administration of the catecholamine uptake blockers bupropion (Randall et al. 2015) and lisdexamfetamine (Yohn et al. 2016b), and the DAT inhibitors MRZ-9547 (Sommer et al. 2014), PRX-14040 (Yohn et al. 2016d), and GBR12909 (Yohn et al. 2016). In contrast, the SERT inhibitor fluoxetine reduces selection of high effort lever pressing in rats responding on a progressive ratio (PROG)/chow feeding choice task at doses that also decrease extracellular DA in nucleus accumbens (Yohn et al. 2016a,e). This is consistent with studies showing that SERT inhibitors decrease high-effort physical activities such as voluntary wheel running (Weber et al. 2009; Claghorn et al. 2016).

Although there are several behavioral tasks that have been used for decades to assess the effects of antidepressant drugs in rodents (e.g. swim test, tail suspension test), none of these procedures specifically targets effort-related decision making, and therefore they do not seem suited for studying the effort-related effects of SERT inhibitors. Moreover, since SERT inhibitors generally yield a positive profile in the traditional behavioral tests related to depression, yet these drugs are relatively ineffective at treating effort-related motivational symptoms in depressed people, it underscores the need to have behavioral tests in animals that specifically focus on the effort-related effects of drugs used to treat depression. Therefore, the goal of these studies was to determine if the 5-$HT_{2C}$ receptor mediates effort-related effects by studying the ability of 5-$HT_{2A}$ and 5-$HT_{2C}$ antagonists to reverse the effects of fluoxetine on effort-related decision making. It was hypothesized that: a) systemic administration of fluoxetine would induce an effort-related impairment in rats performing on the concurrent FR5/chow feeding choice task similar to its effects on PROG/chow lever pressing; b) the reduction in FR5/chow lever pressing induced by fluoxetine would be reversed by antagonism of 5-$HT_{2C}$

receptors, but not 5-$HT_{2A}$ receptors; and c) systemic administration of 5-$HT_{2C}$ receptors would increase high-effort PROG/chow lever pressing.

## 1.2 Materials and Methods

*Animals*

Adult male, drug-naïve, Sprague Dawley rats (Envigo, Indianapolis, IN, USA) were housed in a colony maintained at 23 °C with 12-h light/dark cycles (lights on 07:00). Rats were obtained weighing 275–299 g at the beginning of the study, and were initially food restricted to 85% of their free-feeding body weight for operant training. Rats were allowed modest weight gain throughout the experiment. Supplemental chow was provided as needed to maintain body weights throughout the study, with water available ad libitum. Animal protocols were approved by the University of Connecticut Animal Care and Use Committee, and followed NIH guidelines.

*Behavioral and Pharmacological Methods*

Behavioral sessions were conducted in operant chambers (28 x 23 x 23 cm; Med Associates, Fairfax, VT). Sessions lasted 30 minutes a day for 5 days/week. First, rats were trained to lever press on a continuous reinforcement schedule to receive 45 mg high-carbohydrate pellets (Bio-Serv; Frenchtown, NJ, USA) for one week, then were shifted to the FR5 schedule. After 5 weeks of FR5 training, chow was introduced. During each FR5/chow feeding choice task session, 15-20 g of lab chow (Laboratory Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO) was concurrently available on the floor of the chamber. Rats were trained on this FR5/chow feeding choice procedure for 5 weeks, after which drug testing began. On baseline and drug treatment days, rats consumed all of the operant pellets that were delivered during each session.

All experiments used a within-groups (i.e., repeated measures) design, in which all animals received all treatment combinations in a randomly varied order. During the drug treatment phase, rats were run 5 days per week, with 4 days being baseline days, and one day per week (either Thursday or Friday) being the drug treatment day. In all of the studies listed below, fluoxetine (10.0 mg/kg, 12.5 mg/kg, or 15.0 mg/kg IP) was administered 90 min before testing. Experiment 1 was conducted to establish a dose/response curve for fluoxetine, in which male and female rats were administered vehicle or one dose of fluoxetine (5.0, 10.0, or 15.0 mg/kg) in a randomly varied order over the course of four weeks. For the drug reversal studies, in which one drug is given to induce a behavioral impairment, animals also received a second injection of various doses of either the 5-$HT_{2A}$ antagonist MDL 100907, or the 5-$HT_{2C}$ antagonist SB 242084 or vehicle to attempt to reverse the effects of fluoxetine. Each reversal study would typically involve 5 combined drug treatments (e.g. vehicle/vehicle, fluoxetine/vehicle, and fluoxetine plus 3 doses of the 5-HT antagonist).

List of Drug Experiments:

1. Effect of fluoxetine on effort-related choice.

2. Effect of 5-$HT_{2C}$ antagonist SB 242084 on the effort-related effects of fluoxetine.

3. Effect of 5-$HT_{2A}$ antagonist MDL 100907 on the effort-related effects of fluoxetine.

4. Effect of SB 242084 on performance on the PROG/chow feeding choice task.

5. Effect of MDL 100907 on performance on the PROG/chow feeding choice task.

*Data Analyses*

Repeated measures analysis of variance (ANOVA) was used to determine the effect of drug treatment on lever pressing and chow intake in the behavioral pharmacology experiments. To determine if there were significant reversal effects, nonorthogonal planned comparisons were performed, using the overall error term to assess differences between each treatment and the control condition. The number of comparisons was restricted to the number of treatments minus one (Keppel, 1991).

**1.3 Results**

FLX (5.0-15.0 mg/kg) was administered to male and female rats prior to performance on the FR5/chow feeding choice task. In both groups, FLX significantly reduced lever pressing, but had no significant effect on chow intake. A treatment x sex repeated measures factorial ANOVA revealed a significant overall main effect of treatment on lever pressing [$F(3,42)=29.323$, $p<0.001$], but no effect of sex on lever pressing (p=n.s.), and no significant treatment x sex interaction [$F(3,42)=0.307$, p=n.s.] (**Fig. 1A**). Due to a non-significant interaction, non-orthogonal planned comparisons were computed collapsed across male and female lever presses. It was shown that all three doses of FLX significantly reduced lever presses relative to the vehicle treatment [VEH vs. 5.0 mg/kg FLX, $F(1,42)=16.747$, $p<0.001$; VEH vs. 10.0 mg/kg FLX, $F(1,42)=65.577$, $p<0.001$; VEH vs. 15.0 mg/kg FLX, $F(1,42)=107.601$, $p<0.001$]. Dose of FLX had no effect on chow intake (p=n.s.) (**Fig. 1B**).

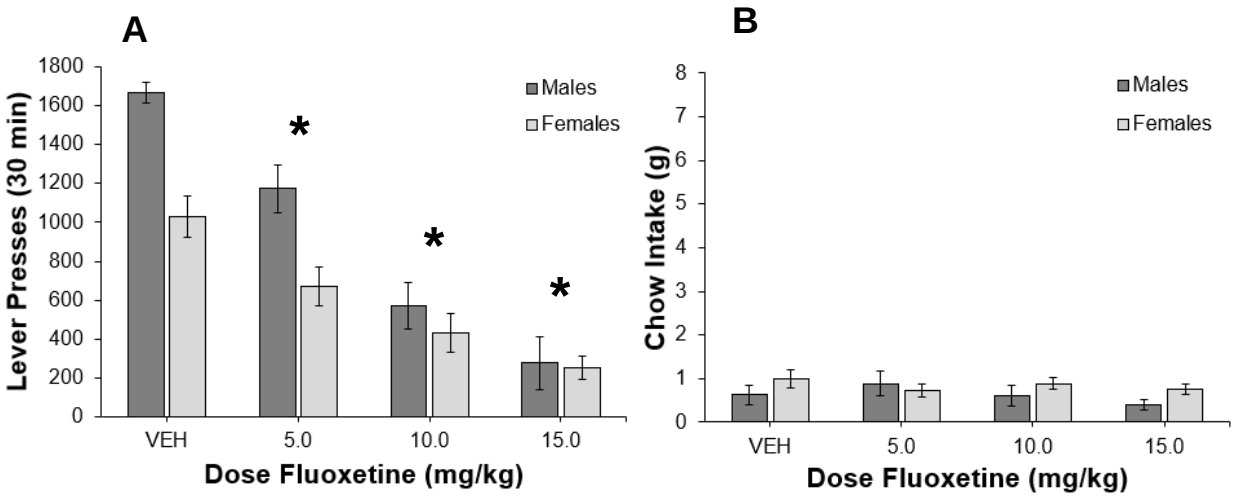



**Figure 1.** Effects of FLX on performance on the FR5/chow feeding choice task in male and female rats. Male (n=8) and female (n=8) rats were injected with vehicle or fluoxetine (5.0-15.0 mg/kg IP). **(A)** Fluoxetine significantly reduced lever pressing at 5.0 mg/kg, 10.0 mg/kg, and 15.0 mg/kg (*p<0.001) and **(B)** had no effect on chow intake (p=n.s.).

A set of reversal experiments were conducted to investigate the effects of 5-$HT_{2C}$ or 5-$HT_{2A}$ receptor antagonists SB 242084 or MDL 100907 on the effort-related impairments induced by fluoxetine (10.0 mg/kg) in male and female rats. The experiments sought to assess the effects of SB 242084 (0.25-2.0 mg/kg) on fluoxetine-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice task. Repeated measures ANOVA demonstrated a significant overall main effect of treatment on lever pressing in males [$F(5,40)=31.076$, $p<0.001$] and females [$F(5,40)=10.249$, $p<0.001$] (**Fig. 2A**). Planned comparisons revealed a significant reduction of lever presses in the FLX plus vehicle condition compared to the vehicle plus vehicle condition in males [$F(1,40)=82.101$, $p<0.001$] and females [$F(1,40)=23.883$, $p<0.001$]. Co-administration of FLX with SB 242084 did not produce a significant reversal from the FLX plus vehicle condition at any of the doses tested (p=n.s.). There was no significant overall effect of treatment on chow intake (p=n.s.) (**Fig. 2B**).

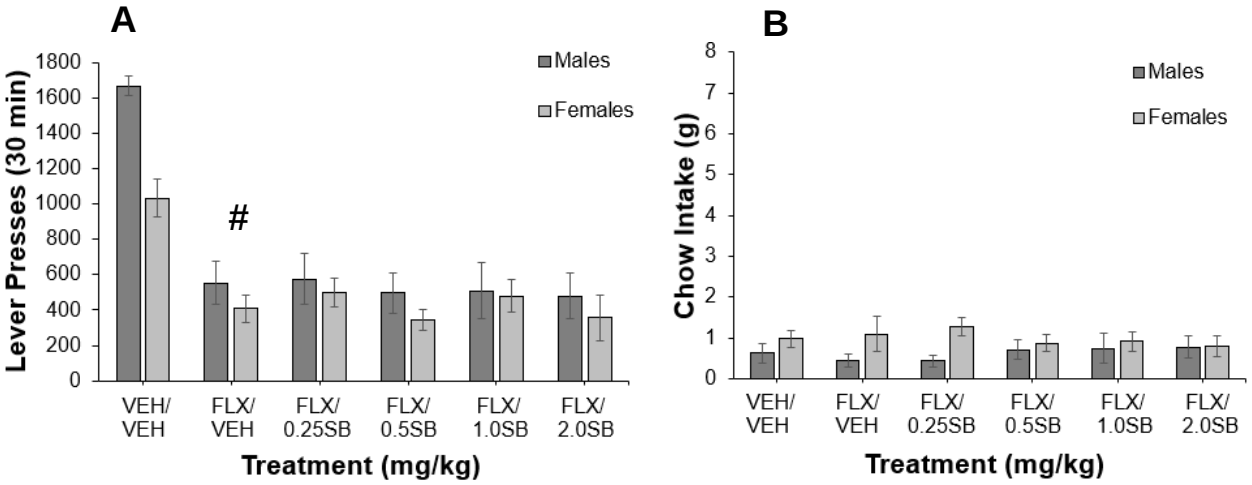



**Figure 2.** The effects of SB 242084 on fluoxetine-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure in male and female rats. Male (n=8) and female (n=8) rats were injected with vehicle or fluoxetine (10.0 mg/kg IP) and vehicle or one of four doses of SB 242084 (0.25 mg/kg, 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg IP). **(A)** Fluoxetine significantly reduced lever pressing (#p<0.001) and **(B)** had no effect on chow intake relative to vehicle, but there was no significant reversal with SB 242084 at any dose.

The second reversal experiment was conducted to investigate the effects of the 5-$HT_{2A}$ receptor antagonist MDL 100907 on the effort-related impairments induced by fluoxetine (15.0 mg/kg) in male and female rats on the concurrent FR5 lever pressing/chow feeding choice task. Repeated measures ANOVA demonstrated a significant overall main effect of treatment on lever pressing in males [F(5,25)=46.214, p<0.001] and females [F(5,25)=19.362, p<0.001] (**Fig. 3A**). Planned comparisons revealed a significant reduction of lever presses in the FLX plus vehicle condition compared to the vehicle plus vehicle condition in males [F(1,25)=46.214, p<0.001] and females [F(1,25)=55.168, p<0.001]. Co-administration of FLX with SB 242084 at any of the doses tested did not produce a significant reversal from the FLX plus vehicle condition (p=n.s.). There was no significant overall effect of treatment on chow intake (p=n.s.) (**Fig. 3B**).

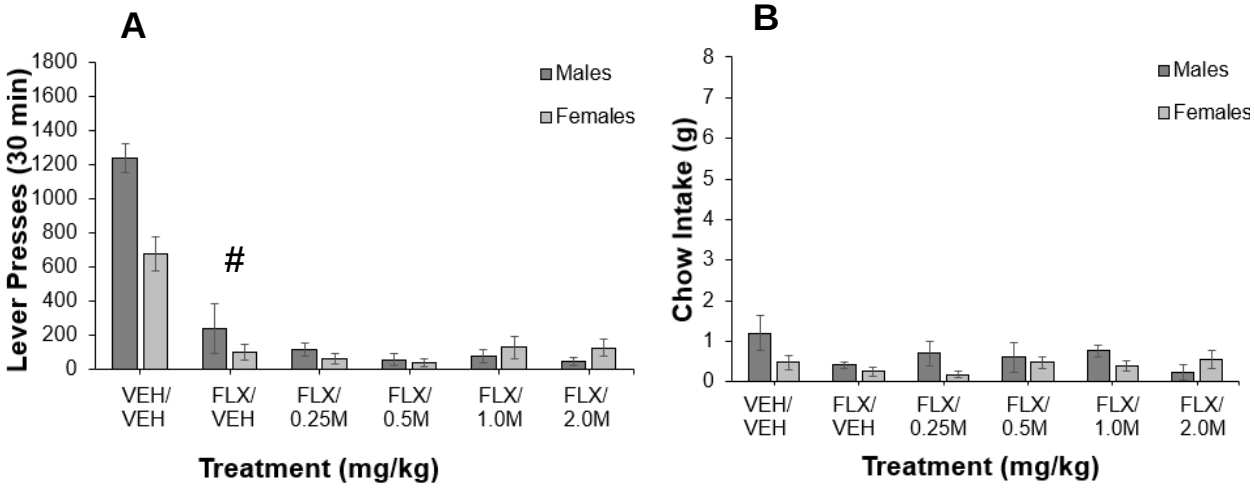


**Figure 3.** The effects of MDL 100907 on fluoxetine-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure in male and female rats. Male (n=6) and female (n=8) rats were injected with vehicle or fluoxetine (15.0 mg/kg IP) and vehicle or one of four doses of MDL 100907 (0.25 mg/kg, 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg IP). **(A)** Fluoxetine significantly reduced lever pressing (#p<0.001) and **(B)** had no effect on chow intake relative to vehicle, but there was no significant reversal with MDL 100907 at any dose.

Additional experiments investigated the ability of SB 242084 to increase lever pressing on the PROG/chow feeding choice task in male and female rats. Vehicle or one of four doses of SB 242084 (0.5-4.0 mg/kg) was administered to rats prior to task performance. Repeated measures ANOVA revealed that SB 242084 did not have a significant overall effect on lever pressing in males [F(4,28)=0.742, p=n.s.] or females [F(4,28)=0.514, p=n.s.] (**Fig. 4A**). In addition, there was no significant overall effect of treatment on chow intake (p=n.s.). Tests of between-subjects effects revealed a significant overall difference in chow intake between male and female rats [F(1,14)=146.536, p<0.001]. (**Fig. 4B**).

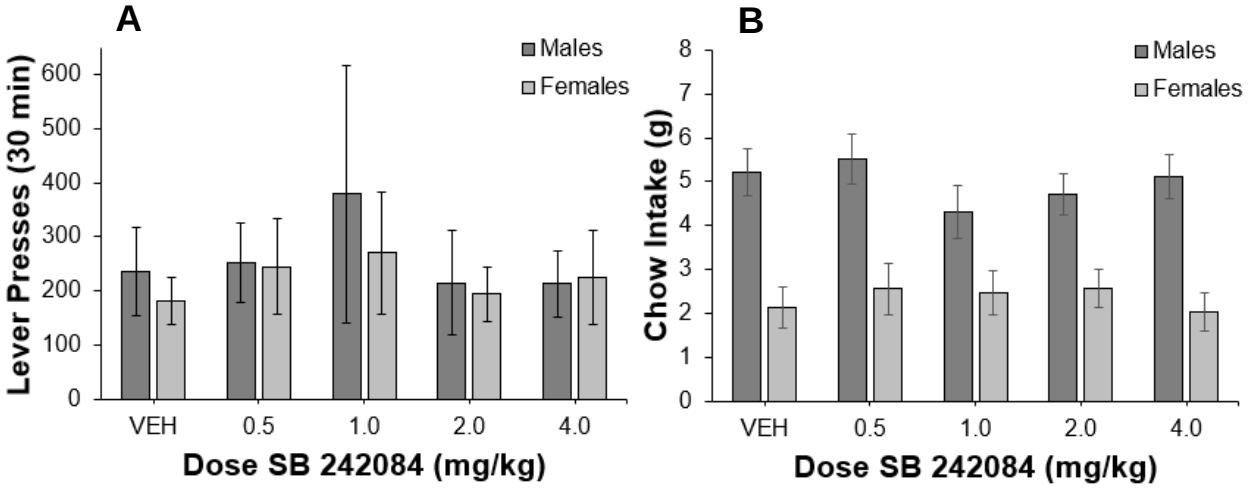



**Figure 4.** The effects of SB 242084 on effort-related performance on the concurrent PROG lever pressing/chow feeding choice procedure in male and female rats. Male (n=8) and female (n=8) rats were injected with vehicle or one of four doses of SB 242084 (0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 4.0 mg/kg IP). **(A)** Treatment with SB did not significantly affect lever pressing (p=n.s.) or **(B)** chow intake relative to vehicle (p=n.s.). Chow intake significantly differed between male and female rats (p<0.001).

The fifth experiment in this series assessed the ability of MDL 100907 to increase lever pressing on the PROG/chow feeding choice task in male and female rats. Vehicle or one of four doses of MDL 100907 (0.5-4.0 mg/kg) was administered to rats prior to task performance. Repeated measures ANOVA revealed that MDL 100907 did not have a significant overall effect on lever pressing in males [F(4,28)=1.253, p=n.s.] or females [F(4,28)=0.802, p=n.s.] (**Fig. 5A**). In addition, treatment did not produce a significant effect on chow intake in either group (p=n.s.).

Tests of between-subjects effects revealed a significant overall difference in chow intake between male and female rats [$F(1,14)=43.131$, $p<0.001$]. (**Fig. 5B**).

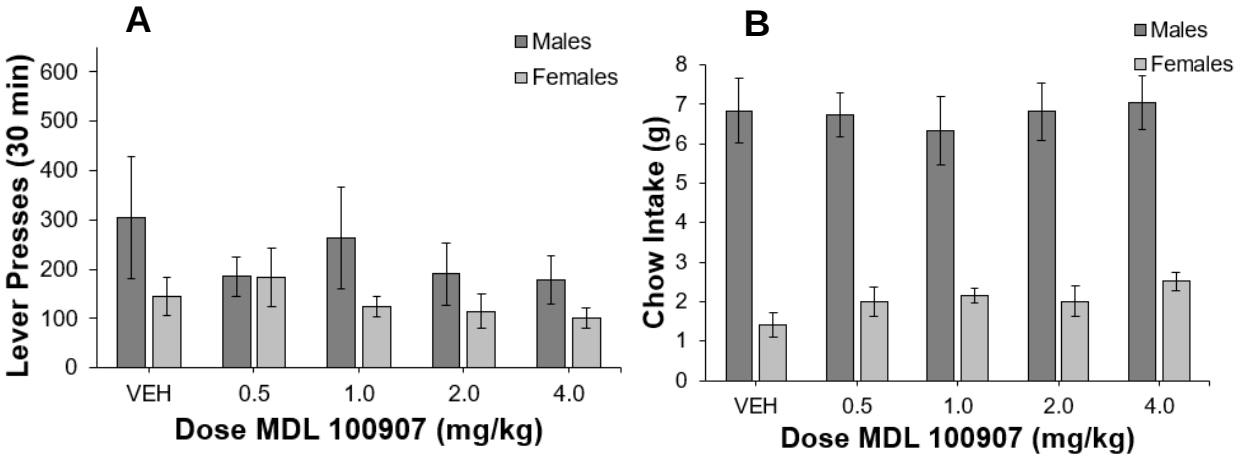



**Figure 5.** The effects of MDL 100907 on effort-related performance on the concurrent PROG lever pressing/chow feeding choice procedure in male and female rats. Male (n=8) and female (n=8) rats were injected with vehicle or one of four doses of MDL 100907 (0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 4.0 mg/kg IP). **(A)** MDL did not significantly affect lever pressing (p=n.s.) or **(B)** chow intake relative to vehicle at any of the doses tested (p=n.s.). Chow intake significantly differed between male and female rats ($p<0.001$).

### 1.4 Discussion

The purpose of Appendix 1 experiments was to evaluate the effort-related effects of the SERT inhibitor fluoxetine, and to assess two different selective 5-HT receptor antagonists for their ability to reverse fluoxetine-induced motivational impairments. Consistent with previous findings from our laboratory using the PROG/chow feeding choice task (Yohn et al. 2016e), fluoxetine administration caused a dose-dependent decrease in lever presses on the FR5/chow feeding choice task. The Yohn et al. (2016e) study was conducted in male rats, while the present experiment used both males and females. In contrast to the effects of DA antagonists or depletions on the FR5/chow task performance to produce compensatory increases in chow consumption, fluoxetine did not have any effect on the amount of chow consumed during the FR5/chow feeding choice task. Though the lack of compensatory chow intake is thought to be a result of possible appetite suppression effects, it is important to note that these effects were not simply due to the use of food reinforcement. Recent studies in our lab demonstrated that systemic fluoxetine administration dose-dependently decreases both running wheel activity and chow consumption in choice procedures in male and female rats (Presby et al., in preparation). In agreement with the data presented here, antagonism of 5-$HT_{2C}$ or 5-$HT_{2A}$ receptors did not reverse the fluoxetine-induced suppression of wheel running or chow intake, nor did they increase high-effort responding when administered alone to rats trained on the PROG/chow procedure. However, the inability of the 5-$HT_{2C}$ receptor antagonist SB 242084 to reverse the

fluoxetine-induced effects is inconsistent with previous literature demonstrating that SB 242084 blocked the rate decreasing effect of fluoxetine on lever pressing (Bauer et al. 2015), and also increased lever pressing (Martin et al. 2002). Antagonism of 5-$HT_{2C}$ receptors increased burst firing in ventral tegmental area (VTA) DA neurons (DiMatteo et al. 1999; DiGiovanni et al. 1999), increased accumbens DA release (Visser et al. 2015), and blocked the decrease in DA neuron activity induced by the SERT inhibitor citalopram (Dremenecov et al. 2009), which would have suggested that blocking this receptor might have been able to reverse the effects of fluoxetine. This inconclusive pattern of effects may be due in part to possible off-target peripheral actions of fluoxetine and SB 242084 related to systemic administration. Thus, it may be beneficial to replicate these studies using intracranial administration of selective 5-HT antagonists into areas of the brain implicated in motivation and mesolimbic DA transmission such as the VTA. In view of the fact that SERT inhibitors are relatively ineffective at treating effort-related motivational symptoms of depression, and that these drugs often induce or exacerbate these debilitating symptoms, ongoing and future work in this area could contribute significantly to the understanding of the neurotransmitter interactions that underlie the motivational effects of antidepressants, and could foster the development of agents that would improve motivational function in people treated with SERT inhibitors.

**Appendix 2: Assessment of pimavanserin in animal models of effort-related choice behavior: significance for treating motivational dysfunctions.**

## 2.1 Introduction

Behavioral activation and effort-related processes are fundamental aspects of motivation. Considerable evidence from the basic neuroscience literature indicates that nucleus accumbens dopamine (DA) is an important component of the neural circuitry that regulates behavioral activation and effort-related aspects of motivation (Salamone et al. 2007, 2016a,b). Studies of behavioral activation and effort also are clinically relevant. In humans, symptoms such as anergia, psychomotor retardation, amotivation and fatigue reflect pathologies in behavioral activation. These symptoms are fundamental features of depression, Parkinson's disease and schizophrenia (Tylee et al. 1999; Stahl 2002; Demyttenaere et al. 2005; Salamone et al. 2006, 2007, 2012; Chong et al. 2015). Within the last few years, rodent procedures assessing effort-related choice behavior have been developed into formal animal models that are useful for characterizing the effects of well-known and experimental therapeutic agents.

Tests of effort-based choice behavior allow animals to select between a more preferred reward that can only be obtained by high exertion of effort vs. a low effort/low reward option. In our laboratory, several tasks have been developed for assessing effort-related choice behavior in rats. Our most commonly used task is an operant concurrent choice task that offers rats a choice between lever pressing to obtain a relatively preferred food (high carbohydrate pellets), vs. approaching and consuming a less preferred food (lab chow) that is concurrently available. Under non-drug conditions, rats lever pressing on a fixed ratio 5 (FR5) schedule typically get most of their food by lever pressing, and they eat only small amounts of chow. With rats performing on this task, pre-feeding to reduce food motivation was shown to suppress both lever pressing and chow intake (Salamone et al. 1991). In contrast, low-to-moderate doses of DA antagonists produced a very different pattern of effects. DA antagonists with varying degrees of receptor subtype specificity, including cis-flupenthixol, haloperidol, raclopride, eticlopride, SCH 23390, SKF83566 and ecopipam, all decreased lever pressing for food but substantially increased intake of the concurrently available chow (Salamone et al. 1991, 2002; Nowend et al. 2001; Sink et al. 2008). The low dose of haloperidol that produced this effect (0.1 mg/kg) did not alter food intake or preference in free-feeding choice tests (Salamone et al. 1991). In addition, it has been demonstrated that intra-accumbens administration of the muscarinic acetylcholine receptor agonist pilocarpine shifted rats' behavior on the FR5/chow task to the low-effort bias, seen as a reduction of lever presses and increased selection of freely available chow (Nunes et al. 2013b). Although DA antagonists and the muscarinic agonist pilocarpine have been shown to reduce FR5 lever pressing and increase chow intake, appetite suppressants such as fenfluramine and cannabinoid CB1 antagonists (Salamone et al., 2002; Sink et al. 2008) do not increase chow intake at doses that suppress lever pressing.

In addition to the FR5/chow feeding task, a progressive ratio (PROG)/chow feeding choice task has been developed. With this task, the rats have a choice between lever pressing vs. approaching and consuming the freely available chow, however, the PROG lever pressing schedule is more demanding because the ratio requirement gets progressively higher throughout the session (Randall et al. 2012). Thus, rats tested on this procedure generally press less and each

chow more than they do on the FR5/choice procedure, because the increasing PROG work demand makes the animals switch from lever pressing to chow intake once the ratio requirement gets high enough (Randall et al. 2012). As with the other effort-based tasks, administration of the DA antagonists haloperidol, ecopipam, and eticlopride all decrease selection of the high-effort PROG lever pressing at doses that do not impair chow intake (Randall et al. 2012, 2014).

More recently, models have been established that assess the effects of the VMAT-2 inhibitor tetrabenazine. This drug depletes DA by blocking vesicular storage, and tetrabenazine is useful for animal models because it produces depressive symptoms in people (Guay 2010). At low doses that reduce extracellular DA and alter DA-related signal transduction in nucleus accumbens, tetrabenazine also alters effort-based choice in animals responding on multiple tasks, including the FR5/chow feeding choice task described above (Nunes et al. 2013), the PROG/chow feeding choice task (Randall et al. 2014), and the T-maze barrier choice task (Yohn et al. 2015a,b). Tetrabenazine induced these effort-related effects at doses that did not alter food intake, food preference, sucrose preference, hedonic reactivity to sucrose, or reference memory. Together with the other results reviewed above, these findings demonstrate that interference with DA transmission does not simply reduce appetite for food or food-related hedonia, but rather, alters effort-based choice and produces a bias away from high-effort activity towards low-effort activity.

Over the last few years, several drugs have been assessed for their ability to improve effort-related performance in these models. The adenosine $A_{2A}$ receptor antagonists istradefylline, Lu AA47070, MSX-3 and MSX-4 have all been shown to reverse the effort-related effects of DA antagonists and tetrabenazine across multiple tasks (Farrar et al. 2007, 2010; Worden et al. 2009; Mott et al. 2009; Salamone et al. 2009; Nunes et al. 2010; Collins et al. 2011; Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2015a). DA $D_1$ receptor agonists also reversed the effort-related effects of DA antagonism and depletion in rats tested on the FR5/chow feeding choice and T-maze barrier choice tasks (Yohn et al. 2015b). Recent studies demonstrated that the glycine uptake inhibitor bitopertin was able to reverse the effort-related effects of haloperidol (Yohn et al. 2017). In addition, several drugs that block DA uptake, including bupropion, GBR12909, lisdexamfetamine, PRX-14040, methylphenidate, and modafinil, all were able to reverse the effort-related effects of tetrabenazine (Nunes et al. 2013; Randall et al. 2014; Yohn et al. 2016a,b,c; Salamone et al. 2016). Moreover, in otherwise untreated rats that were tested on the PROG/chow feeding choice task, the DA transport inhibitors bupropion, GBR12909, lisdexamfetamine and PRX-14040 all were shown to increase selection of the high-effort PROG lever pressing and decrease choice of the low effort option (chow intake; Randall et al. 2015; Yohn et al. 2016b,c,d). These results demonstrate the feasibility of using tests of effort-based choice for preclinical characterization of compounds with different pharmacological profiles, and also emphasize the role of brain DA systems as a modulator of effort-related functions. This animal research demonstrating the involvement of DA systems in effort-related aspects of motivation is consistent with studies showing that effort-based decision making in humans is related to DA transmission (Treadway et al. 2011; Wardle et al. 2012), and that motivational function can be improved in depressed patients by administration of drugs that increase DA transmission (Stotz et al. 1998; Papakostas et al. 2006). Furthermore, the use of effort-based tasks in rodents as models of human psychopathology is validated by studies showing that a low-effort bias on choice tasks is seen in human patients with major depressive disorder (Treadway et al. 2012b; Yang et al. 2014, 2015), schizophrenia (Gold et al.

2013, 2015; Reddy et al. 2015) and Parkinson's disease (Chong et al. 2015). Nevertheless, it is worth pointing out that these animal models have limitations, and do not directly model effort-based tasks in human subjects suffering from depression, schizophrenia, Parkinson's disease or other disorders.

In contrast to the effects of drugs that act to increase DA transmission, drugs that block serotonin (5-HT) transport, such as the SSRIs fluoxetine and citalopram, failed to reverse the effects of tetrabenazine on FR5/chow feeding choice performance (Yohn et al. 2016a,b). Furthermore, fluoxetine and citalopram suppressed lever pressing in rats tested on the FR5/chow feeding choice task (Yohn et al. 2016a,b), and fluoxetine reduced PROG lever pressing after both acute and repeated administration (Yohn et al. 2016e). Fluoxetine also was shown to decrease extracellular DA in nucleus accumbens at a dose that suppressed PROG lever pressing (Yohn et al. 2016e). These results are consistent with human clinical studies reporting that SSRIs are relatively poor at treating motivational dysfunctions in depressed people (Papakostas et al. 2006; Bell et al. 2013; Fava et al. 2014; Rothschild et al. 2014; Cooper et al. 2014). In fact, these drugs can actually worsen effort-related symptoms such as fatigue.

Because of the widespread prevalence of motivational dysfunctions related to problems with behavioral activation and effort, which are evident across multiple disorders, it is critical to develop a line of preclinical research for assessing the effects of potential treatments. If elevations in 5-HT transmission due to uptake blockade are relatively ineffective at restoring normal motivational function, and in fact can impair effort-related processes, it is critical to study drugs that blunt 5-HT transmission to determine if they can exert pro-motivational effects. In view of the ability of pimavanserin to act as an inverse agonist at the $5\text{-HT}_{2A}$ receptor, it is important to assess the effects of pimavanserin in animal models of effort-related choice behavior. Pimavanserin (also known as ACP-103) has been approved for human use in treating hallucinations and delusions associated with Parkinson's disease (trade name: Nuplazid®). Previous research has shown that pimavanserin has anti-tremor effects in an animal model of Parkinsonian tremor (Vanover et al. 2008), but its effects on effort-related motivational processes are unknown.

This project was undertaken determine if pimavanserin exert effects in animal models of effort-related choice behavior, and can reverse the effects of the SSRI fluoxetine and the antipsychotic drugs haloperidol and risperidone, as well as the DA depleting agent tetrabenazine, on performance of these tasks. In the initial experiments, pimavanserin was assessed for its ability to reverse the effects of the serotonin transport (SERT) inhibitor fluoxetine in rats tested on the FR5/chow feeding choice task, and additional experiments studied the ability of pimavanserin to reverse the effects of the DA $D_2$ antagonist haloperidol, as well as the atypical antipsychotic risperidone, and the muscarinic agonist pilocarpine, on the FR5/choice procedure. Finally, pimavanserin and MDL 100907 were tested for their ability to reverse the effort-related effects of tetrabenazine.

## 2.2 Materials and Methods

*Animals*

Adult male, drug-naïve, Sprague Dawley rats (Envigo, Indianapolis, IN, USA) were housed in a colony maintained at 23 °C with 12-h light/dark cycles (lights on 07:00). Rats were obtained weighing 275–299 g at the beginning of the study, and were initially food restricted to 85% of their free-feeding body weight for operant training. Rats were fed supplemental chow to maintain weight throughout the study, with water available ad libitum. Rats were allowed modest weight gain throughout the experiment. Animal protocols were approved by the University of Connecticut Animal Care and Use Committee, and followed NIH guidelines.

*Behavioral and Pharmacological Methods*

Behavioral sessions were conducted in operant chambers (28 x 23 x 23 cm; Med Associates, Fairfax, VT). Sessions lasted 30 minutes a day for 5 days/week. First, rats were trained to lever press on a continuous reinforcement schedule to receive 45 mg high-carbohydrate pellets (Bio-Serv; Frenchtown, NJ, USA) for one week, then were shifted to the FR5 schedule. After 5 weeks of FR5 training, chow was introduced. During each FR5/chow feeding choice task session, 15-20 g of lab chow (Laboratory Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO) was concurrently available on the floor of the chamber. Rats were trained on this FR5/chow feeding choice procedure for 5 weeks, after which drug testing began. On baseline and drug treatment days, rats consumed all of the operant pellets that were delivered during each session.

All experiments used a within-groups (i.e., repeated measures) design. Thus, once animals were trained for a given experiment, all animals received all treatment combinations in a randomly varied order. During the drug treatment phase, rats were run 5 days per week, with 4 days being baseline (drug-free) days, and one day per week (either Thursday or Friday) being the drug treatment day. All of the studies listed below (except Exp 3) are drug reversal studies, in which one drug is given to induce a behavioral impairment (12.5 mg/kg IP fluoxetine 90 min before testing, 0.1 mg/kg IP haloperidol 50 min before testing, 0.5 mg/kg IP risperidone 30 min before testing, and 1.0 mg/kg IP tetrabenazine 120 min before testing). Animals also received a second injection of various doses of pimavanserin or MDL 100907 to attempt to reverse the effects of the first drug. Each study would typically involve 5 combined drug treatments (e.g. vehicle/vehicle, haloperidol/vehicle, and haloperidol plus 3 doses of pimavanserin).

<u>List of Drug Experiments</u>

1. FR5/chow feeding with pimavanserin vs. fluoxetine
2. FR5/chow feeding with pimavanserin vs. haloperidol
3. FR5/chow feeding with risperidone (to determine effective dose)
4. FR5/chow feeding with pimavanserin vs. risperidone
5. FR5/chow feeding with pimavanserin vs. pilocarpine

6. FR5/chow feeding with pimavanserin vs. tetrabenazine

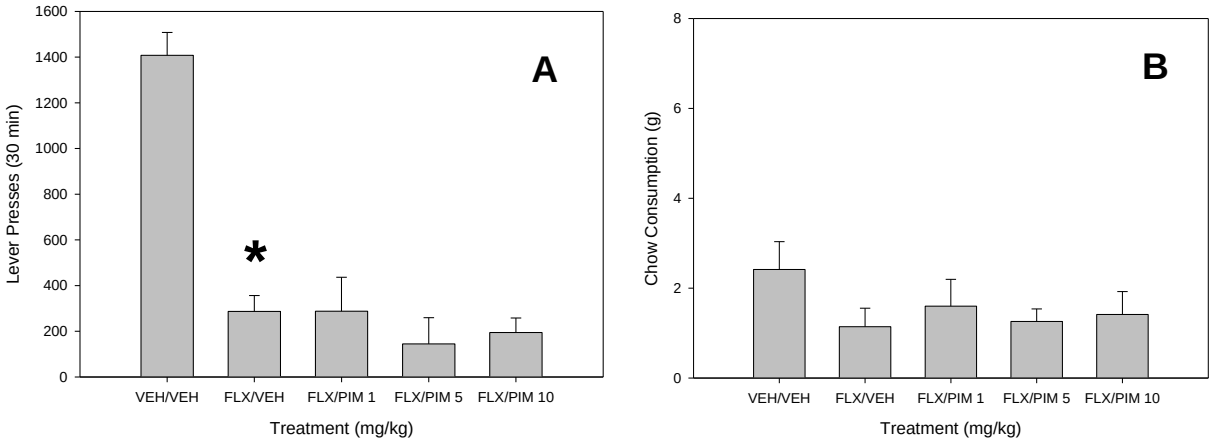
7. FR5/chow feeding with MDL 100907 vs. tetrabenazine

*Data Analyses*

Repeated measures analysis of variance (ANOVA) was used to determine the effect of drug treatment on lever pressing and chow intake in the behavioral pharmacology experiments. To determine if there were significant reversal effects, nonorthogonal planned comparisons were performed, using the overall error term to assess differences between each treatment and the control condition. The number of comparisons was restricted to the number of treatments minus one (Keppel, 1991).

## 2.3 Results

A series of experiments were conducted to investigate the effects of the 5-$HT_{2A}$ inverse agonist pimavanserin on the effort-related impairments induced by various pharmacological challenges. The first set of experiments sought to assess the effects of pimavanserin on fluoxetine-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice task. Repeated measures ANOVA demonstrated a significant overall main effect of treatment on lever pressing [$F(4,24)=25.603$, $p<0.001$] (**Fig. 1A**), [$F(4,20)=16.527$, $p<0.001$] (**Fig. 1C**). There was no overall effect of drug treatment on chow intake in either experiment. Planned comparisons indicated that fluoxetine (12.5 mg/kg) significantly reduced lever pressing compared to the vehicle condition ($p<0.001$; **Fig. 1A and 1C**), but had no effect on chow intake (**Fig. 1B, 1D**). There was no significant reversal of lever pressing when pimavanserin was co-administered at any dose. Furthermore, the route of administration (subcutaneous vs. intraperitoneal) had no effect on the ability of pimavanserin to reverse the fluoxetine-induced suppression of lever pressing.



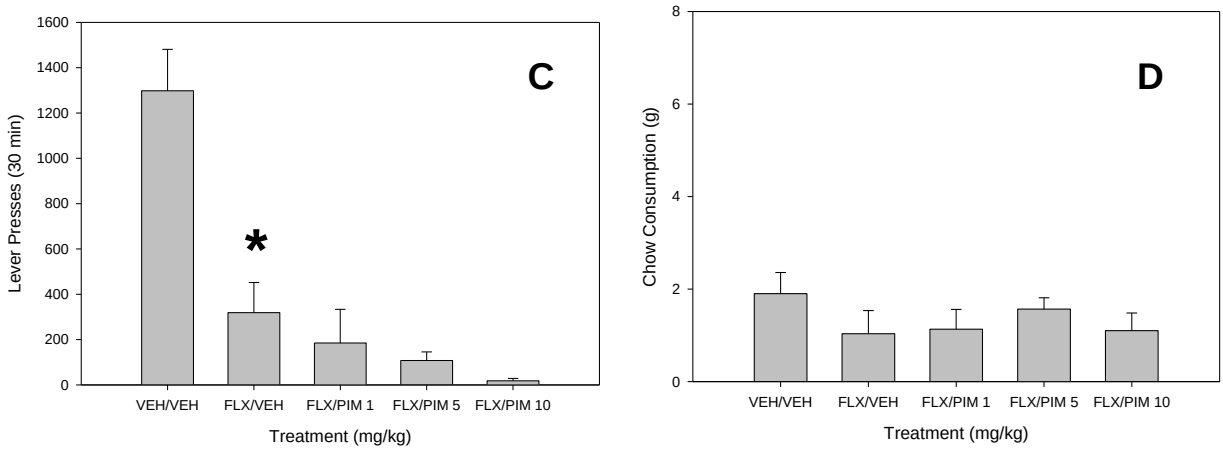



**Figure 1.** The effects of pimavanserin on fluoxetine-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure. **(A,B)** Fluoxetine and pimavanserin (subcutaneous; SC). Rats (n=7) were injected with vehicle or fluoxetine (12.5 mg/kg IP) and vehicle or one of three doses of pimavanserin (1.0, 5.0, or 10.0 mg/kg SC). **(A)** Fluoxetine significantly reduced lever pressing (*$p<0.001$) and **(B)** had no effect on chow intake, but there was no significant reversal with pimavanserin at any dose. **(C,D)** Fluoxetine and pimavanserin (intraperitoneal; IP). Rats (n=6) were injected with vehicle or fluoxetine (12.5 mg/kg IP) and vehicle or one of three doses of pimavanserin (1.0, 5.0, or 10.0 mg/kg IP). **(C)** Fluoxetine significantly reduced lever pressing (*$p<0.001$) from vehicle, and **(D)** had no effect on chow intake, but there was no significant reversal with pimavanserin at any dose.

**Figure 2** shows the effects of pimavanserin on haloperidol-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure. Repeated measures ANOVA revealed a significant overall main effect of treatment on lever pressing and chow intake in all three experiments (**Fig. 2A:** [$F(4,28)=13.575$, $p<0.001$]; **Fig. 2B:** [$F(4,28)=5.079$, $p<0.05$]; **Fig. 2C:** [$F(4,28)=22.012$, $p<0.001$]; **Fig. 2D:** [$F(4,28)=13.653$, $p<0.001$]; **Fig. 2E:** [$F(3,21)=66.199$, $p<0.001$]; **Fig. 2F:** [$F(3,21)=25.642$, $p<0.001$]. Planned comparisons were performed and showed that haloperidol (0.1 mg/kg) significantly reduced lever pressing ($p<0.001$) and increased chow intake ($p<0.001$) in all three experiments (**Fig. 2**). There was no significant reversal of the haloperidol-induced effort-related effects on lever pressing or chow intake by pimavanserin at any dose, regardless of route of administration.

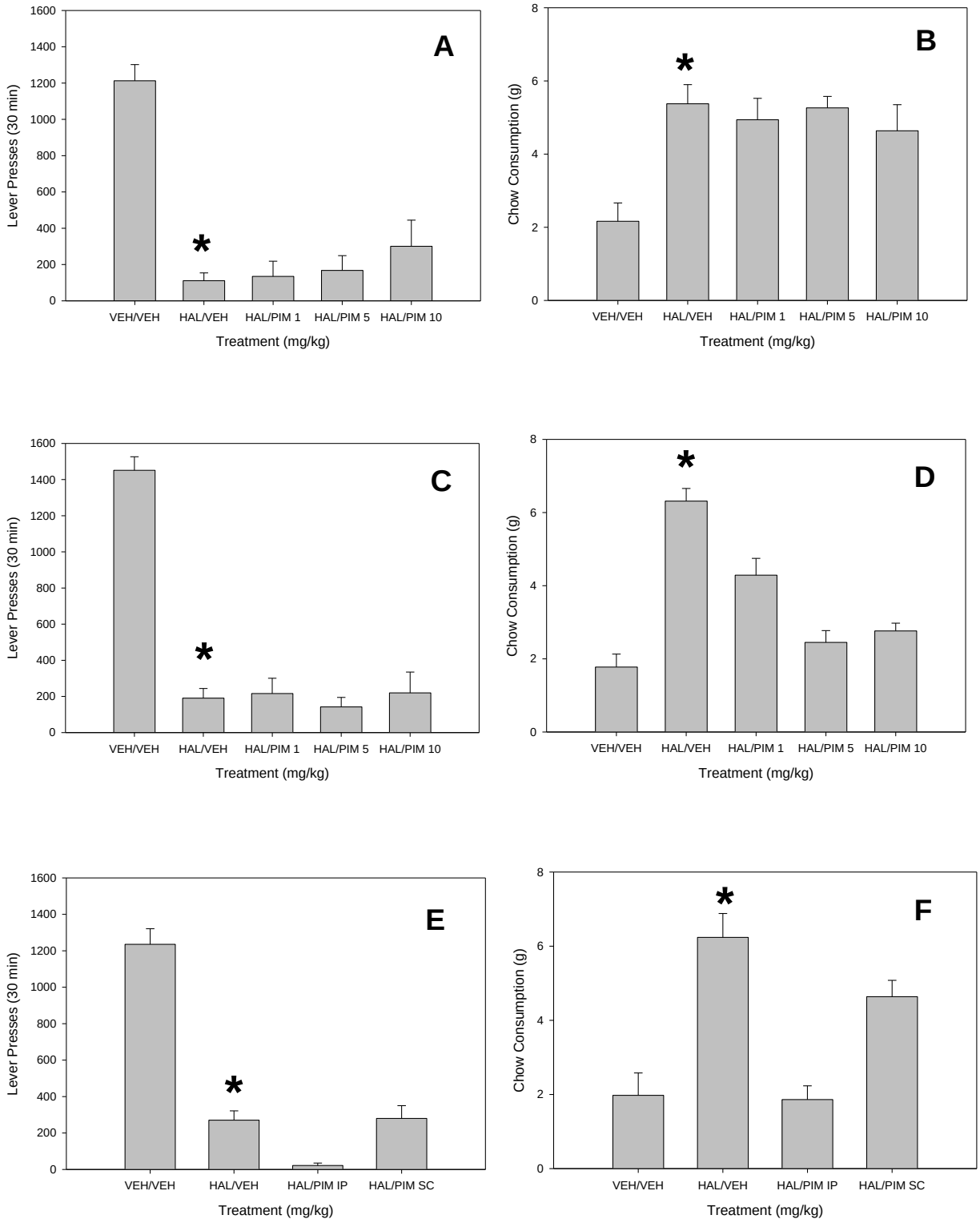



**Figure 2.** The effects of pimavanserin on haloperidol-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure. **(A,B)** Haloperidol and

pimavanserin (subcutaneous; SC). Rats (n=8) were injected with vehicle or haloperidol (0.1 mg/kg IP) and vehicle or one of three doses of pimavanserin (1.0, 5.0, or 10.0 mg/kg SC). **(A)** Haloperidol significantly reduced lever pressing (*p<0.001) and **(B)** increased chow intake (*p<0.001) from vehicle, but there was no significant reversal with pimavanserin at any dose. **(C,D)** Haloperidol and pimavanserin (intraperitoneal; IP). Rats (n=8) were injected with vehicle or haloperidol (0.1 mg/kg IP) and vehicle or one of three doses of pimavanserin (1.0, 5.0, or 10.0 mg/kg IP). **(C)** Haloperidol significantly reduced lever pressing (*p<0.001) and **(D)** increased chow intake from vehicle, but there was no significant reversal with pimavanserin. **(E,F)** Haloperidol and pimavanserin (IP vs. SC) at a higher dose. Rats (n=8) were injected with vehicle or haloperidol (0.1 mg/kg IP) and vehicle or pimavanserin (20 mg/kg IP or SC). **(E)** Haloperidol significantly reduced lever pressing (*p<0.001) and **(F)** increased chow intake (*p<0.001) from vehicle, but there was no significant reversal with pimavanserin using either IP or SC routes of administration.

The antipsychotic drug risperidone was administered to rats trained on the FR5/chow feeding choice procedure to evaluate its effects effort-related choice behavior, and to determine the effective dose to be used to induce a motivational deficit in pharmacological challenge reversal experiments. Repeated measures ANOVA showed a significant effect of treatment on lever pressing [$F(5,35)=25.758$, $p<0.001$] and chow intake [$F(5,35)=11.525$, $p<0.001$]. When administered alone, risperidone significantly reduced lever pressing at 0.25 mg/kg ($p<0.001$) and 0.5 mg/kg ($p<0.001$) (**Fig. 3A**), and significantly increased chow intake at 0.5 mg/kg ($p<0.001$) (**Fig. 3B**).

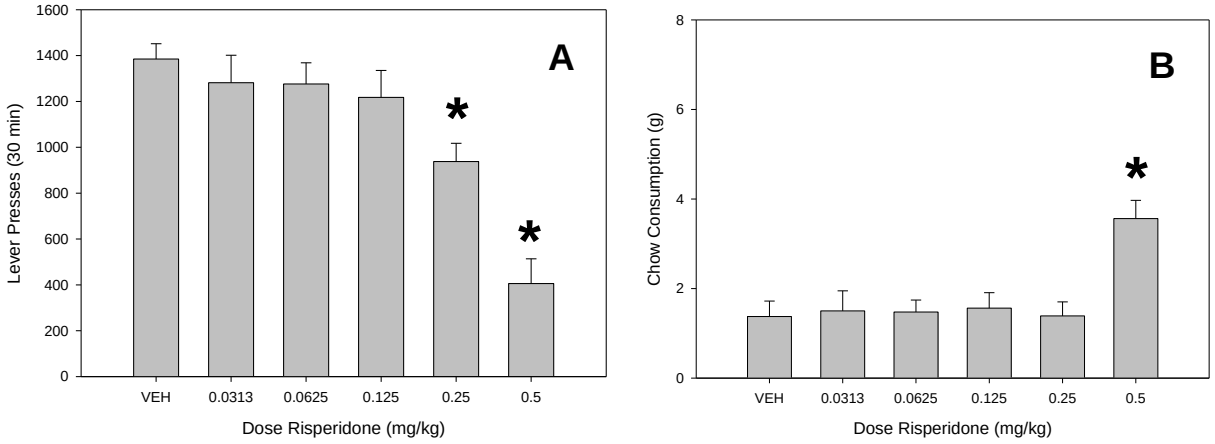



**Figure 3.** The effects of risperidone when administered alone to rats on the concurrent FR5 lever pressing/chow feeding choice procedure. Rats (n=8) were injected with vehicle or risperidone (0.0313-0.5 mg/kg IP). **(A)** Risperidone significantly reduced lever presses at 0.25 mg/kg (*p<0.001) and 0.5 mg/kg (*p<0.001). **(B)** Risperidone significantly increased chow intake at 0.5 mg/kg (*p<0.001).

In a fourth experiment, pimavanserin was assessed for its ability to reverse the suppression of lever pressing caused by risperidone. There was a significant overall main effect of drug treatment on lever pressing [$F(4,60)=53.847$, $p<0.001$], but no significant effect of drug treatment on chow intake [$F(4,60)=0.636$, n.s.]. Risperidone induced a significant reduction of lever pressing on the FR5/chow feeding choice task compared to the vehicle/vehicle condition (**Fig. 4A**), but had no effect on chow intake (**Fig. 4B**). Co-administration of pimavanserin (1.0-10.0 mg/kg) with risperidone did not produce a significant reversal of the reduction in lever pressing at any dose.

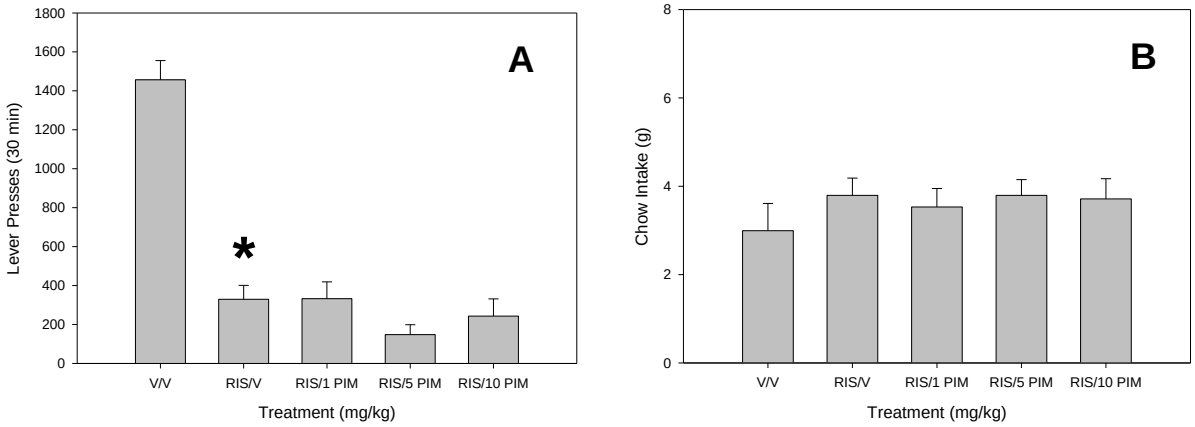



**Figure 4.** The effects of pimavanserin on risperidone-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure. Rats (n=16) were injected with vehicle or risperidone (0.5 mg/kg IP) and vehicle or one of three doses of pimavanserin (1.0, 5.0, or 10.0 mg/kg SC). **(A)** Risperidone significantly reduced lever pressing from vehicle/vehicle (*$p<0.001$) but there was no significant reversal with pimavanserin at any dose. **(B)** There was no significant effect of risperidone or pimavanserin on chow intake.

**Figure 5** shows the effects of pimavanserin on pilocarpine-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice task. There was a significant overall main effect of treatment on lever pressing [$F(3,21)=28.287$, $p<0.001$], and no effect of drug treatment on chow intake [$F(3,21)=0.656$, n.s.]. Pilocarpine administration caused a reduction in lever pressing ($p<0.001$) (**Fig. 5A**), but did not affect chow intake (**Fig. 5B**). Pimavanserin did not cause a reversal of pilocarpine-induced suppression of lever pressing at either dose.

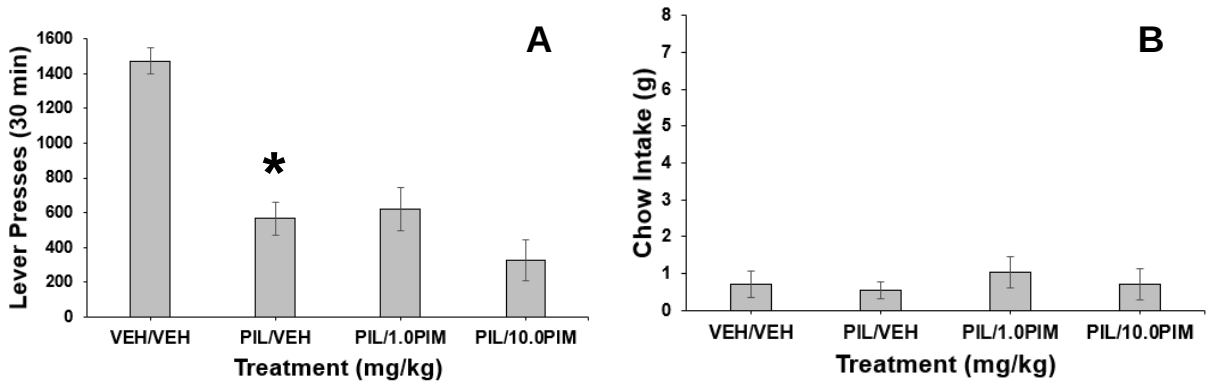



**Figure 5.** The effects of pimavanserin on pilocarpine-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure. Rats (n=8) were injected with vehicle or pilocarpine (1.0 mg/kg IP) and vehicle or one of two doses of pimavanserin (1.0 or 10.0 mg/kg SC). **(A)** Pilocarpine significantly reduced lever pressing from vehicle/vehicle (*p<0.001) and **(B)** had no effect on chow intake. There was no significant reversal of the pilocarpine-induced suppression of lever pressing with pimavanserin at any dose.

The DA-depleting agent tetrabenazine was used to induce an effort-related impairment in rats on the FR5/chow feeding choice task in the final two experiments. As shown in **Figure 6**, a repeated measures ANOVA demonstrated a significant overall main effect of drug treatment on lever pressing [F(4,60)=45.831, p<0.001] and on chow intake [F(4,60)=16.782, p<0.001]. Tetrabenazine significantly reduced lever presses (p<0.001) (**Fig. 6A**) and increased chow intake (**Fig. 6B**) compared to the vehicle treatment. These effects were not attenuated by pimavanserin at any dose.

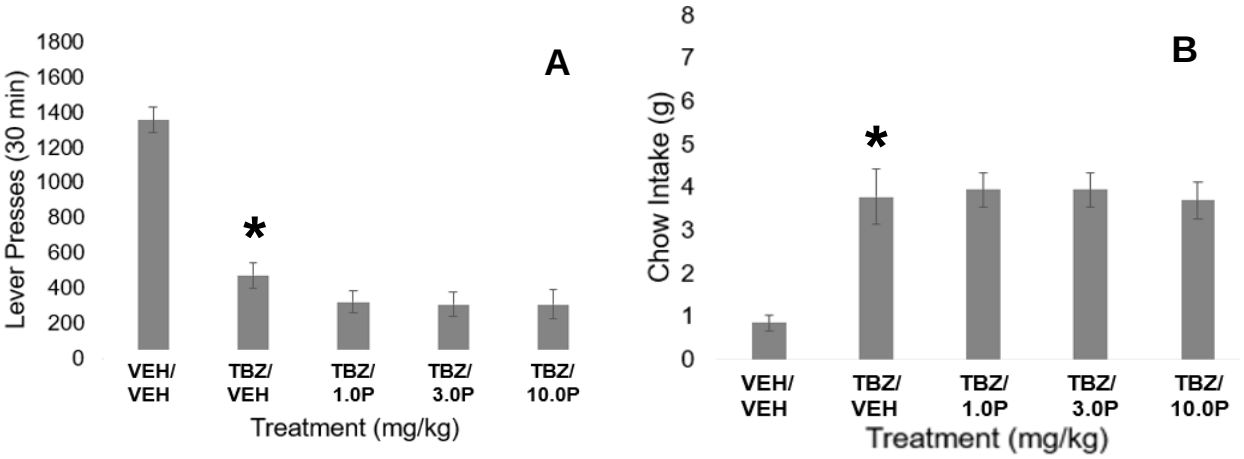



**Figure 6.** The effects of pimavanserin on tetrabenazine (TBZ)-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure. Rats (n=16) were injected

with vehicle or tetrabenazine (1.0 mg/kg IP) and vehicle or one of three doses of pimavanserin (1.0, 5.0 mg/kg, or 10.0 mg/kg SC). **(A)** Tetrabenazine significantly reduced lever pressing (*p<0.001) and **(B)** increased chow intake (*p<0.001) from vehicle/vehicle, but there was no significant reversal of the tetrabenazine-induced effects on lever pressing or chow intake with pimavanserin at any dose.

The last experiment of the series assessed whether the tetrabenazine-induced effects on lever pressing and chow intake could be reversed by the co-administration of MDL 100907. A one-way ANOVA revealed a significant reduction of lever presses when tetrabenazine and MDL were co-administered compared to the tetrabenazine plus vehicle treatment group [F(1,31)=4.458, *p<0.05] (**Fig. 7A**). Co-administration of tetrabenazine and MDL did not differ from the tetrabenazine plus vehicle treatment condition (**Fig. 7B**).

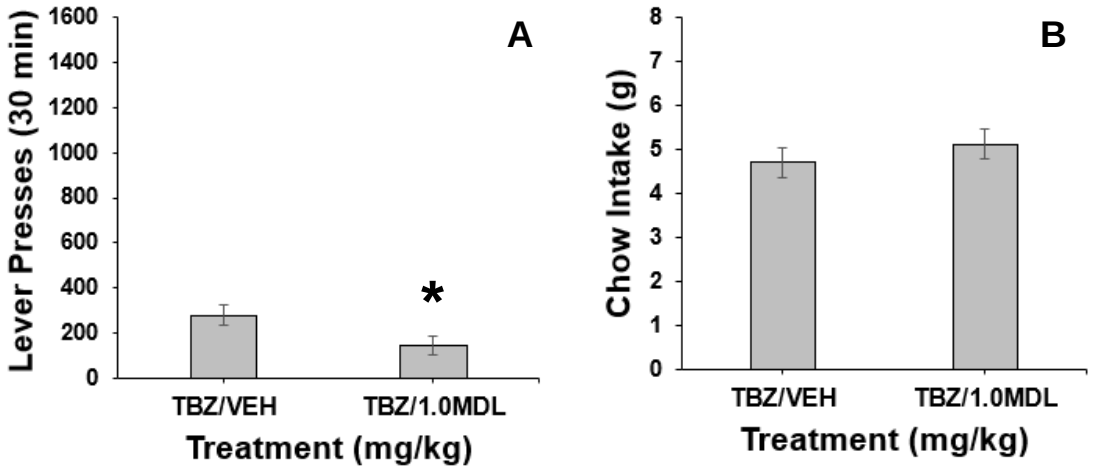



**Figure 7.** The effects of MDL 100907 on tetrabenazine-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure. Rats (n=16) were injected with tetrabenazine (1.0 mg/kg IP) and MDL 100907 (1.0 mg/kg IP). **(A)** One-way ANOVA revealed a significant reduction of lever presses when TBZ and MDL were co-administered compared to the TBZ plus vehicle treatment group [F(1,31)=4.458, *p<0.05]. **(B)** Chow intake was unaffected [F(1,31)=0.753, n.s.].

## 2.4 Discussion

Across all 6 reversal experiments, pimavanserin showed no signs of reversing the effects of fluoxetine, haloperidol, risperidone, pilocarpine, or tetrabenazine. It seems unlikely that these negative results were due a lack of range of doses tested, because depending upon the experiment, pimavanserin doses ranging from 1.0 mg/kg up to 20.0 mg/kg were used. Furthermore, it was determined that the route of drug administration did not have an effect on the

results. These results are consistent with other data (Rotolo Ph.D. Dissertation Appendix 1) indicating that the selective 5-$HT_{2A}$ antagonist MDL 100907 and the 5-$HT_{2B}$ antagonist SB 242084 also were ineffective in at improving performance in fluoxetine-treated rats tested on effort-based choice procedures. Experiment 7 showed that MDL 100907 also was unable to reverse the effects of tetrabenazine. Taken together, these results suggest that the tested drugs that act upon 5-$HT_2$ family receptors were ineffective at reversing any of the drug-induced impairments in effort-based choice induced by a variety of different drugs with distinct pharmacological profiles (e.g. SERT inhibitor, DA antagonists, DA depleting agent, muscarinic agonist). The present results should be put into context by recognizing the limitations of the methods being used. The effort-based choice models employed may not predict accurately the effects of serotonergic drugs in human subjects suffering from psychiatric disorders like Parkinson's disease, schizophrenia or depression. Nevertheless, these negative results serve to emphasize the important role that dopaminergic drugs have in modulating effort-based choice behavior (e.g. Rotolo et al. 2019).

**Appendix 3: Assessment of the role of kappa opioid receptor function in animal models of effort-related choice behavior: significance for treating motivational dysfunctions.**

## 3.1 Introduction

Individuals that suffer from major depressive disorder (MDD) and other psychiatric disorders often experience an array of symptoms affecting mood, cognition, and motivation. The most commonly used antidepressant drug therapies include selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants, but these drugs do not mitigate some of the most debilitating symptoms, and are often accompanied by negative side effects (Montgomery et al. 1994; Li et al. 2016). Moreover, individuals that are treated with DA antagonists or depleting agents often experience effects such as depression, anergia and motivational dysfunctions (Frank 2009, 2010; Guay, 2010; Chen et al. 2012; Kennedy et al. 2014). Drugs that inhibit the uptake of the dopamine (DA) transporter (DAT) have demonstrated clinical efficacy in reducing some of the physical symptoms of depression, but can be accompanied by adverse effects such as psychostimulant effects, psychosis, and abuse liability, limiting their therapeutic use. The drawbacks of the currently approved pharmacological treatments beg the need for the investigation of an alternative class of drugs that will more effectively treat symptoms of amotivation in humans.

Since most of the widely prescribed antidepressants affect some aspect of monoamine transmission, there may be utility in assessing compounds in a distinct category for their safety and efficacy as treatments for depression. Recently, it has been noted that the endogenous opioid system has been implicated in some regulatory aspects of MDD symptoms. There are several different modulators of the endogenous opioid system that have been characterized by distinct structures and patterns of functional activity (Waldhoer et al. 2004; Guerrero et al. 2019). The kappa opioid receptor (KOR) has been identified as a key central nervous system modulator and is distributed throughout the brain, including in areas relevant in disorders such as depression and schizophrenia (Simonin et al. 1995; Mague et al. 2003; Carlezon et al. 2006; Ranganathan et al. 2012). Some preclinical and clinical evidence suggests that KOR antagonists have therapeutic value for stress- and mood-related symptoms of depression and anxiety disorders (Mague et al. 2003; Shippenberg 2009; Lalanne et al. 2014), but the ability of this class of drugs to ameliorate symptoms of motivational dysfunction and fatigue have not been explored.

Animal studies of effort-related choice behavior are being used to model effort-related motivational dysfunctions in humans. Our laboratory has developed several behavioral tasks in rodents that assess the role of DA, serotonin (5-HT), adenosine, and GABA in the exertion of effort and effort-related decision making (Salamone and Correa 2002; Salamone et al. 1991, 2012). With these procedures, animals are offered a choice between high effort instrumental actions leading to highly valued reinforcers vs. low effort/low reward options. Several previous studies have shown that DAT inhibitors, including GBR12909, lisdexamfetamine, methylphenidate, and PRX-14040, can reverse the effort-related effects of the vesicular monoamine transport inhibitor tetrabenazine, which blocks DA storage (Nunes et al. 2013, Yohn et al. 2016a,b,d; Salamone et al. 2016b). Other studies have demonstrated that SERT inhibitors such as fluoxetine (Prozac) and citalopram (Lexapro) are not effective at treating fatigue and anergia, and can even exacerbate them (Cooper et al. 2014; Yohn et al. 2016a,b,d). Because

many drugs that block DAT or SERT produce a number of undesirable side effects, there is a need to investigate novel alternative or adjunctive drug therapies, such as modulators of the endogenous opioid system, to aid in the treatment of effort-related symptoms.

The purpose of these studies was to investigate the function of the KOR in effort-related aspects of motivation. Initial experiments sought to determine if a novel KOR antagonist (BlackThorn Therapeutics) can reverse impairments in effort-related choice behavior induced by the dopamine (DA) storage blocker tetrabenazine and the DA $D_2$ receptor antagonist/5-$HT_{2A}$ receptor inverse agonist risperidone. Additional experiments investigated the effects of the benzodiazepine inverse agonist FG-7142 on effort-based choice as a comparison to the effects induced by DA depletion or antagonism. Finally, the ability of the KOR antagonist to reverse the effects of FG-7142 was assessed.

## 3.2 Materials and Methods

*Animals*

Adult male, drug-naïve, Sprague Dawley rats (Envigo, Indianapolis, IN, USA) were housed in a colony maintained at 23 °C with 12-h light/dark cycles (lights on 07:00). Rats were obtained weighing 275–299 g at the beginning of the study, and were initially food restricted to 85% of their free-feeding body weight for operant training. Rats were fed supplemental chow to maintain weight throughout the study, with water available ad libitum. Rats were allowed modest weight gain throughout the experiment. Animal protocols were approved by the University of Connecticut Animal Care and Use Committee, and followed NIH guidelines.

*Behavioral and Pharmacological Methods*

Behavioral sessions were conducted in operant chambers (28 x 23 x 23 cm; Med Associates, Fairfax, VT). Sessions lasted 30 minutes a day for 5 days/week. First, rats were trained to lever press on a continuous reinforcement schedule to receive 45 mg high-carbohydrate pellets (Bio-Serv; Frenchtown, NJ, USA) for one week, then were shifted to the FR5 schedule. After 5 weeks of FR5 training, chow was introduced. During each FR5/chow feeding choice task session, 15-20 g of lab chow (Laboratory Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO) was concurrently available on the floor of the chamber. Rats were trained on this FR5/chow feeding choice procedure for 5 weeks, after which drug testing began. On baseline and drug treatment days, rats consumed all of the operant pellets that were delivered during each session.

All experiments used a within-groups (i.e., repeated measures) design. Thus, once animals were trained for a given experiment, all animals received all treatment combinations in a randomly varied order. During the drug treatment phase, rats were run 5 days per week, with 4 days being baseline (drug-free) days, and one day per week (either Thursday or Friday) being the drug treatment day. Three of the studies listed below (Experiments 1, 2, and 4) are drug reversal studies, in which one drug is given to induce a behavioral impairment (1.0 mg/kg IP tetrabenazine 120 min before testing, 0.5 mg/kg IP risperidone 30 min before testing, and 6.0 mg/kg IP FG-7142 30 min before testing). Animals also received a second injection of various doses of vehicle or the KOR antagonist (3.0, 10.0, or 30.0 mg/kg P.O. 120 min before testing) to

attempt to reverse the effects of the first drug. Each study would typically involve 5 combined drug treatments (e.g. vehicle/vehicle, tetrabenazine/vehicle, and tetrabenazine plus 3 doses of KOR antagonist). Experiment 3 was conducted to establish a dose/response curve for the FG-7142, in which animals were administered vehicle or one dose of FG-7142 (2.0, 4.0, 6.0, or 8.0 mg/kg) in a randomly varied order over the course of five weeks.

List of Drug Experiments:

1. Effect of KOR antagonist on the effort-related effects of tetrabenazine.

2. Effect of KOR antagonist on the effort-related effects of risperidone.

3. Effect of FG-7142 on effort-related choice.

4. Reversal of the effort-related effects of the benzodiazepine inverse agonist FG-7142 by KOR antagonist.

*Data Analyses*

Repeated measures analysis of variance (ANOVA) was used to determine the effect of drug treatment on lever pressing and chow intake in the behavioral pharmacology experiments. To determine if there were significant reversal effects, nonorthogonal planned comparisons were performed, using the overall error term to assess differences between each treatment and the control condition. The number of comparisons was restricted to the number of treatments minus one (Keppel, 1991).

### 3.3 Results

*1. Effect of KOR antagonist on the effort-related effects of tetrabenazine.* A series of experiments were conducted to investigate the effects of a recently synthesized KOR antagonist (BlackThorn Therapeutics) on the effort-related impairments induced by various pharmacological challenges. The first experiment sought to assess the effects of the KOR antagonist on tetrabenazine-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice task. Repeated measures ANOVA demonstrated a significant overall main effect of treatment on lever pressing [$F(4,56)=48.295$, $p<0.001$] (**Fig. 1A**) and on chow intake [$F(4,56)=21.927$, $p<0.001$] (**Fig. 1B**). Planned comparisons indicated that tetrabenazine (1.0 mg/kg) significantly reduced lever pressing ($p<0.001$; **Fig. 1A**), and significantly increased chow intake (**Fig. 1B)** compared to the vehicle condition. There was no significant reversal of lever pressing when the KOR antagonist was co-administered at any dose, though chow intake at the two highest doses was significantly lower than tetrabenazine alone.

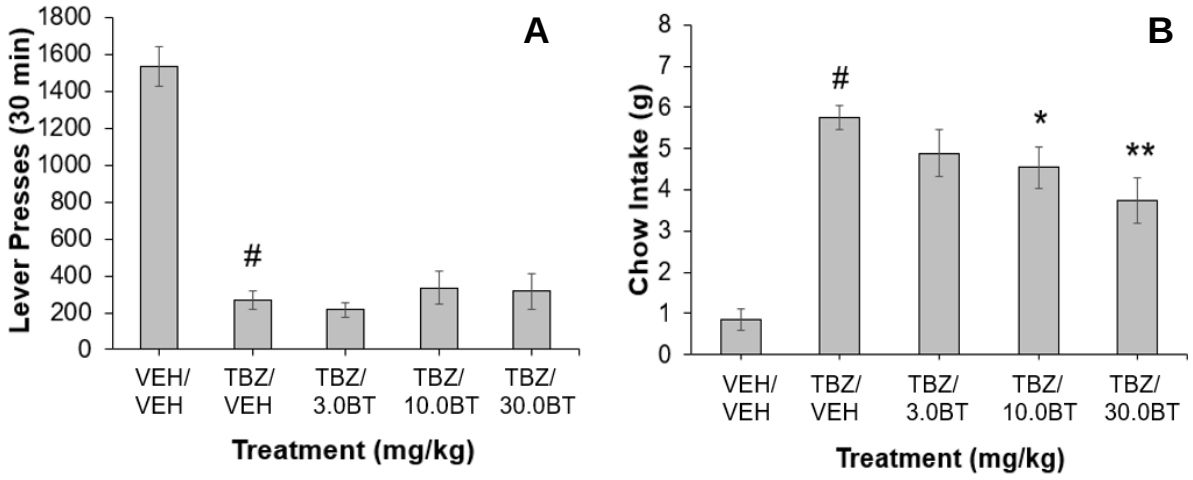



**Figure 1.** The effects of the KOR antagonist (BT) on tetrabenazine-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure. Rats (n=15) were injected with vehicle or tetrabenazine (1.0 mg/kg IP) and vehicle or one of three doses of the KOR antagonist (3.0, 10.0, or 30.0 mg/kg P.O.). **(A)** Tetrabenazine significantly reduced lever pressing (#$p<0.001$) from vehicle. The KOR antagonist BT did not reverse the lever pressing impairment at any dose. **(B)** Tetrabenazine plus vehicle significantly increased chow intake compared to vehicle (#$p<0.001$). Planned comparisons indicated that when co-administered, the KOR antagonist BT significantly reduced chow intake from tetrabenazine plus vehicle at 10.0 mg/kg (*$p<0.05$) and 30.0 mg/kg (**$p<0.001$).

*2. Effect of KOR antagonist on the effort-related effects of risperidone.* **Figure 2** shows the effects of the KOR antagonist on risperidone-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure. Repeated measures ANOVA revealed a significant overall main effect of treatment on lever pressing [$F(4,60)=27.824$, $p<0.001$] (**Fig. 2A**) and chow intake [$F(4,60)=17.350$, $p<0.001$] (**Fig. 2B**). Planned comparisons were performed and showed that risperidone (0.5 mg/kg) significantly reduced lever pressing ($p<0.001$) and increased chow intake ($p<0.001$) compared to vehicle. There was no significant reversal of the risperidone-induced effort-related effects on lever pressing or chow intake by the KOR antagonist at any dose, although there was a trend towards a reversal at 10.0 mg/kg. BT did produce a dose-related reduction in chow intake relative to tetrabenazine alone.

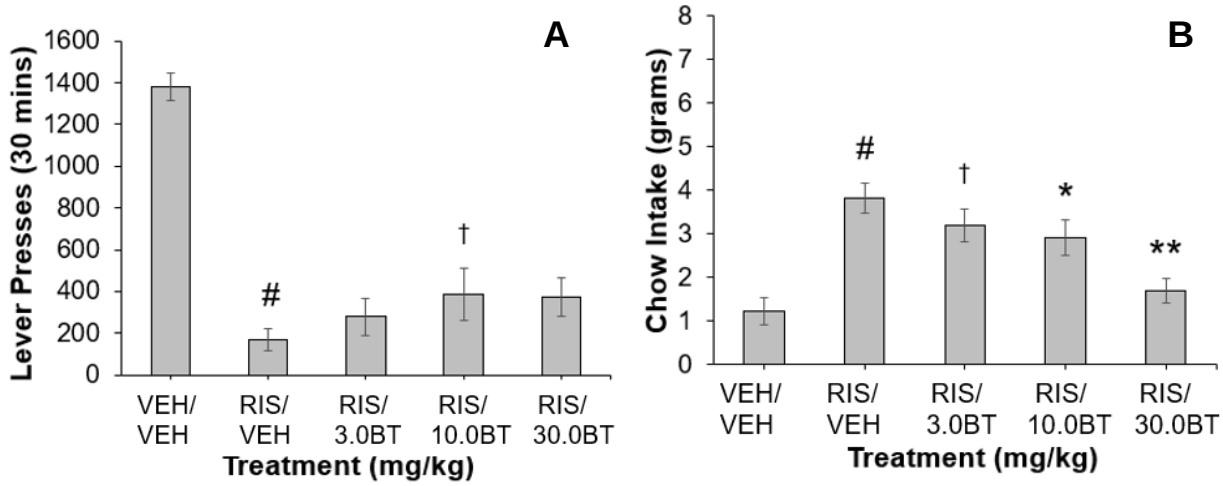



**Figure 2.** The effects of the KOR antagonist (BT) on risperidone-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure. Rats (n=16) were injected with vehicle or risperidone (0.5 mg/kg IP) and vehicle or one of three doses of the KOR antagonist (3.0, 10.0, or 30.0 mg/kg P.O.). **(A)** Risperidone significantly reduced lever pressing (#$p<0.001$) from vehicle. The KOR antagonist BT did not significantly reverse the lever pressing impairment at any dose, however, co-administration of 10.0 mg/kg BT and risperidone approached significance ($^{\dagger}0.05<p<0.1$). **(B)** Tetrabenazine plus vehicle significantly increased chow intake compared to vehicle (#$p<0.001$). Planned comparisons indicated that when co-administered, the KOR antagonist BT significantly reduced chow intake from tetrabenazine plus vehicle at 10.0 mg/kg (*$p<0.05$) and 30.0 mg/kg (**$p<0.001$).

*3. Effect of FG-7142 on effort-related choice.* The benzodiazepine inverse agonist FG-7142 was assessed for its effects on the FR5/chow feeding choice procedure (**Fig. 3**). Repeated measures ANOVA demonstrated a statistically significant overall effect of treatment on lever pressing [$F(4,60)=80.872$, $p<0.001$] (**Fig. 3A**). Planned comparisons revealed a significant reduction in lever presses compared to vehicle at all four doses of FG-7142 (2.0-8.0 mg/kg) ($p<0.001$). Repeated measures ANOVA was also used to assess the effect of FG-7142 on chow intake. Treatment with FG-7142 produced an overall significant effect on chow intake [$F(4,60)=2.544$, $p<0.05$]. (**Fig. 3B**).

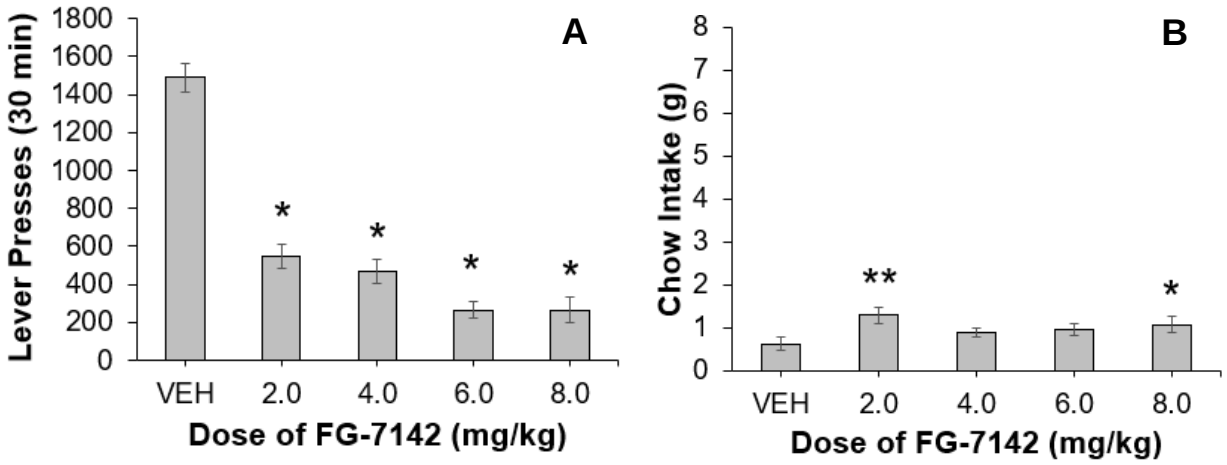



**Figure 3.** The effects of the benzodiazepine inverse agonist FG-7142 on effort-related choice behavior in rats. Rats (n=16) were injected with vehicle or FG-7142 (2.0-8.0 mg/kg IP) prior to performing on the concurrent FR5 lever pressing/chow feeding choice procedure. **(A)** FG-7142 significantly reduced lever pressing (*$p<0.001$) from vehicle at all four doses. **(B)** FG-7142 produced a significant overall effect on chow intake ($p<0.05$). Treatment with 2.0 mg/kg and 8.0 mg/kg significantly increased chow intake relative to vehicle (**$p<0.01$, *$p<0.05$).

*4. Reversal of the effort-related effects of the benzodiazepine inverse agonist FG-7142 by KOR antagonist.* In the fourth experiment, the KOR antagonist was assessed for its ability to reverse the effort-related performance effects of the benzodiazepine inverse agonist FG-7142 in rats performing on the FR5/chow task (**Fig. 4**). Repeated measures ANOVA revealed a significant overall effect of treatment on lever pressing [$F(4,60)=43.362$, $p<0.001$] (**Fig. 4A**). A significant reduction in lever presses was produced by co-administration of 6.0 mg/kg FG-7142 plus vehicle, compared to the vehicle plus vehicle condition. None of the three doses of the KOR antagonist were able to significantly reverse the FG-7142-induced suppression of lever presses. Drug treatment did not have a significant overall effect on chow intake (**Fig. 4B**).

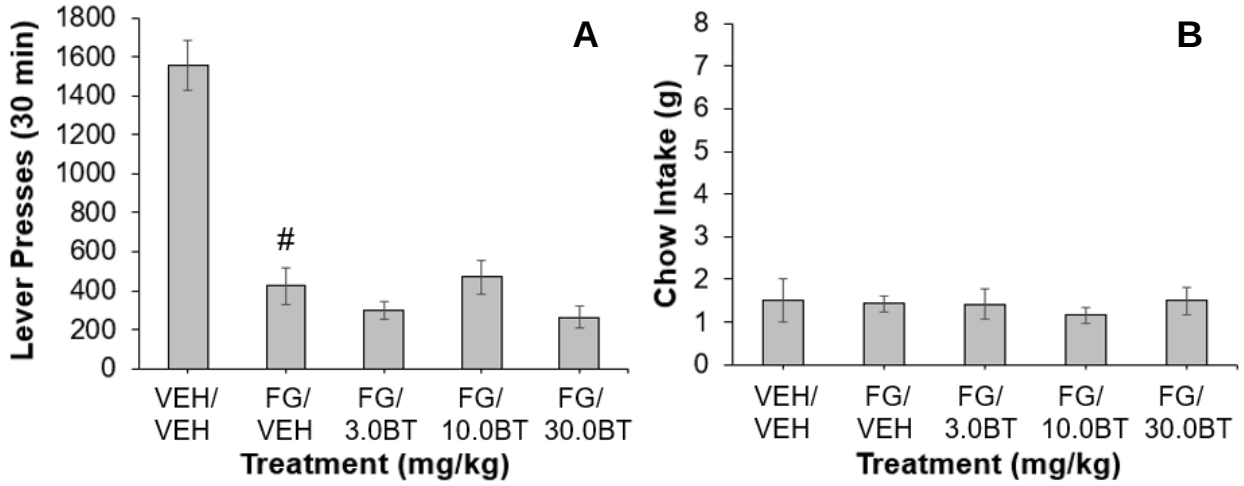



**Figure 4.** Effects of the KOR antagonist (BT) on FG-7142-induced changes in performance on the concurrent FR5 lever pressing/chow feeding choice procedure. Rats (n=16) were injected with vehicle or FG-7142 (6.0 mg/kg IP) and vehicle or one of three doses of the KOR antagonist (3.0, 10.0, or 30.0 mg/kg P.O.). **(A)** FG-7142 significantly reduced lever pressing (#p<0.001) from vehicle. The KOR antagonist BT did not significantly reverse the lever pressing impairment at any dose (p=n.s.). **(B)** Drug treatment did not have an overall effect on chow intake (p=n.s.).

### 3.4 Discussion

The concurrent FR5/chow feeding choice task was useful for assessing the ability of the novel KOR antagonist to reverse the effects of the DA depleting agent tetrabenazine, the DA $D_2$/5-$HT_{2A}$ inverse agonist risperidone, and the benzodiazepine inverse agonist FG-7142. In order to assess the effect of the KOR BT on FG-7142, it also was necessary to determine if FG-7142 induced effort-related effects in rats performing on this task. In summary, the KOR antagonist was unable to reverse the suppression in lever pressing caused by tetrabenazine, risperidone, although it did reverse the increase in chow intake produced by these drugs. BT also failed to reverse the suppression of lever pressing induced by FG-7142. These data suggest that the KOR antagonist is not an effective treatment of effort-related dysfunction when administered on its own as measured by the FR5/chow feeding choice task. Nevertheless, it is possible that it could be tested in the future as a potential adjunct therapy in combination with dopaminergic compounds. Furthermore, previous research has demonstrated that the effects of other KOR antagonists can take up to hours or days (Guerrero et al. 2019), so it is possible that the time course of this more recently synthesized compound may need to be further elucidated in *in vivo* behavioral experiments.

The third experiment provided the first examination of the effects of FG-7142 using a task that assesses effort-based choice. FG-7142 is a benzodiazepine inverse agonist that acts as an anxiogenic drug and produces signs of the stress response. Preclinical studies of the role of stressors in modulating aspects of motivational performance are important, because stress is generally seen as a factor in several psychopathologies. Previous research has shown that restraint stress or administration of the stress-related hormones corticosterone or corticotropin-

releasing hormone can produce a low effort bias in tests of effort-based choice (Shafiei et al. 2012; Bryce and Floresco, 2016; Dieterich et al. 2020). In the present studies, FG-7142 substantially decreased lever pressing, but did not significantly increase chow intake in the dose range tested. Nevertheless, and examination of the dose response curve for FG-7142 indicates that the lowest dose (2.0 mg/kg) produced a substantial reduction in lever pressing, and at that dose there was a tendency for most rats to show an increase in chow intake. Thus, future research should explore the possibility that low doses in the range of 1.0-3.0 mg/kg would in fact produce a significant shift from lever pressing to chow intake.

Taken together, these experiments provide critical preclinical research that address the feasibility of using KOR antagonists to improve effort-related aspects of motivation in people with depression, Parkinson's disease, or schizophrenia.

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